Modeling Sporadic Alzheimer's Disease with Induced Pluripotent Stem Cells and Mechanisms of Aβ-Induced Synaptic Toxicity

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Abstract

Alzheimer's disease (AD) is the most common form of dementia. About 5% of the patients suffer from early onset AD, whereas 95% are diagnosed with late onset, sporadic AD. Thus far, no treatment to slow or halt cognitive decline is available. A deeper understanding of the pathomechanisms of this neurodegenerative disorder is required for an effective treatment. Central to the disease is the accumulation of amyloid-β (Aβ) and tau protein. Aβ aggregates to oligomers and further forms fibrils and plaques. Increased levels of Aβ are thought to initiate the detrimental effects of AD. Widespread changes in all cell types of the brain have been detected. On neurons, the most toxic form of Aβ, soluble oligomers, impair synaptic transmission, mitochondrial function and can ultimately evoke cell death. In AD patient brains mitochondria show increased generation of reactive oxygen species, changes in levels of the five different complexes of the oxidative phosphorylation chain, necessary for ATP production, and altered morphology. Both, synaptic loss and mitochondrial deficits are thought to be evoked by Aβ.

In AD cognitive decline correlates best with synaptic loss. The exact mechanism of synapse loss is not known, but research suggests a pivotal role of the NMDA receptor (NMDAR), which is activated by binding of glutamate and glycine. Aβ-mediated Ca^{2+} influx through aberrant NMDAR activation was thought to lead to long-term depression (LTD) of synapses. However, a metabotropic signaling function of the NDMAR, independent of ion-flux, suggests Ca^{2+} is not required for LTD.

The aim of the first part of this thesis was to gain deeper insight into the mechanism of Aβ induced synaptic loss and the role of metabotropic NMDAR signaling. Analysis of hippocampal slices of a familial AD mouse model, as well as exogenously added Aβ, revealed dendritic spine loss caused by Aβ. Treatment with glutamate binding site antagonist APV rescued spine loss, in contrast to application of MK-801, an open-channel blocker, or Ca^{2+} chelator BAPTA. Aβ-induced NMDAR activation increased p38 MAPK activity, a kinase involved in LTD. Spine loss was prevented by inhibition of p38 MAPK and treatment with APV abolished p38 activation. Inhibition of G protein signaling did not abolish synaptic loss. This suggests that Aβ induces activation of p38 MAPK which leads to synaptic loss, independent of Ca^{2+} influx.

The development of induced pluripotent stem cells (iPSCs) allows the generation of an infinite supply of human material, on the genetic background of an AD patient. In addition, it enables to research AD in human neurons at an early stage of the disease, whereas before only examination of postmortem brain tissue was possible. Thus, it proved to be difficult to delineate cause and consequence of pathological mechanisms.

In the second part of this thesis human iPSC-derived neuronal cells were used to elucidate changes in early AD. A cohort of healthy control subjects and AD patients, characterized by biomarkers, brain imaging and neuropsychology, were recruited and somatic cells reprogrammed to pluripotency. IPSCs were subsequently differentiated into functional induced neurons (iN cells). Neurons from three out five AD patients displayed higher levels of ROS, which suggests mitochondrial dysfunction. Analysis

of oxidative phosphorylation chain complexes revealed increases of complex levels in AD, but no changes in mitochondrial fission and fusion dynamics. As mentioned above, these observed effects may be caused by increased Aβ production, however, no correlation between Aβ and higher ROS generation was found. Further, no difference in total or phosphor-tau levels were observed in iN cells from AD patients. These findings suggest that mitochondrial dysfunction plays an important role in the emergence of an early, presymptomatic phase of AD prior to amyloid and tau aggregation.

Zusammenfassung

Die Alzheimer-Krankheit ist die häufigste Form der Demenz. Ungefähr 5% der Patienten erkranken vor dem 65. Lebensjahr an einer vererblichen Art mit frühem Krankheitsbeginn, wohingegen die restlichen 95% an der sporadischen Alzheimer-Krankheit leiden. Bis heute ist keine Behandlungsmethode verfügbar, um den geistigen Verfall zu verlangsamen oder zu stoppen. Für eine effektive Behandlung ist es notwendig, ein tiefergehendes Verständnis der pathologischen Veränderungen zu erlangen. Die Proteine Amyloid-β (Aβ) und Tau spielen eine zentrale Rolle im Krankheitsverlauf. Aβ aggregiert zu Oligomeren, weitere Aggregation führt zu Fibrillen und aus diesen bestehen Plaques. Es wird angenommen, dass eine erhöhte Menge an Aβ über einen langen Zeitraum zum Ausbruch der Krankheit führt. Die pathologischen Veränderungen betreffen alle Zelltypen des Gehirns. Freie Aβ Oligomere sind die toxischsten Aggregate und führen in Neuronen unter anderem zur Beeinträchtigung der synpatischen Kommunikation sowie mitochondrialer Funktionen und können letztlich zum Zelltod führen. Im erkrankten Gehirn produzieren Mitochondrien erhöhte Werte reaktiver Sauerstoffspezies, unterliegen morphologischen Veränderungen und zeigen Abweichungen in den fünf Komplexen der Atmungskette, welche für die Produktion von ATP verantwortlich ist. Es wird angenommen, dass sowohl der Verlust von Synapsen, wie auch mitochondirale Defizite durch Aβ hervorgerufen wird.

Die kognitiven Defizite der Patientten korrelieren am besten mit dem Verlust von Synapsen. Die Ursache hierfür ist nicht exakt erforscht, es wird jedoch vermutet, dass der NMDA Rezeptor (NMDAR) eine essentielle Rolle in diesem Mechanismus besitzt. Die Aktivierung des Rezeptors erfolgt durch das Binden von Glutamat und Glyzin. Es wurde angenommen, dass Aβ-induzierte abnormale Aktivierung zu Calciumeinstrom führt, welches zu Langzeitdepression von Synapsen führt. Jedoch besitzt der NMDAR Rezeptor auch eine metabotrope Funktion, welche unabhängig von Calcium zur synaptischen Depression führen kann. Die Rolle der metabotropen NMDAR Funktion für den Synapsenverlust bei der Alzheimer`schen Krankheit ist allerdings noch unklar. Der erste Teil dieser Arbeit fokussiert sich darauf, ein tiefergehendes Verständnis des Verlusts von Synapsen und der Rolle der metabotropen Funktion des NMDARs in diesem Prozess zu erlangen. Hippocampale Schnitte von einem Alzheimer Mausmodell, sowie die Applikation von exogenem Aβ zeigen den Verlust von Synapsen. Dies konnte verhindert werden durch Behandlung mit einem Antagonisten der Glutamatbindestelle, wohingegen die Blockade des Calciumkanals des Rezeptors und das Binden freien Calciums Synapsenverlust nicht unterbinden. In diesem Prozess aktiviert Aβ eine Kinase, die auch in klassischer, Caclium-abhängiger synaptischer Depression aktiviert wird. Dies lässt vermuten, dass für den Verlust von Synapsen in der Alzheimer‘schen Krankheit Aβ zur Aktivierung einer Kinase führt, welche einen Signalweg in Gang setzt und zum Verlust von Synapsen führt. Dies geschieht unabhängig von Calciumeinstrom, wie zuvor angenommen.

Eine Limitierung bei der Erforschung der Alzheimer‘schen Krankheit und des Gehirns im generellen, war der Mangel von humanem Material. Dies wurde posthum untersucht, Forschung an lebenden

Neuronen war jedoch bisher nicht möglich. Dies änderte sich mit der Technologie der induzierten pluripotenten Stammzellen (iPSCs), welche es ermöglicht, humane Neurone in einer frühen präsymptomatischen Phase der Krankheit zu untersuchen.

Im zweiten Teil dieser Arbeit werden Neurone aus iPSCs von sporadischen Alzheimer Patienten genutzt um eine frühe Phase der Krankheit zu erforschen. Patienten und gesunde Subjekte wurden mit Hilfe von Biomarkern, bildgebender Gehirnuntersuchung und neurospychologischen Tests charakterisiert. Hautzellen dieser Personen wurden zu Stammzellen reprogrammiert und weiter in funktionelle induzierte Neurone differenziert. Neurone von drei von fünf Patienten zeigten erhöhte Produktion reaktiver Sauerstoffspezies, welches auf dysfunktionale Mitochondrien hinweist. Analysis der verschiedenen Komplexe der mitochondrialen Atmungskette zeigte erhöhte Mengen der Komplexe der Atmungskette, jedoch keine Hinweise auf morphologische Veränderungen der Mitochondrien. Wir fanden keine Korrelation zu veränderten Aβ oder tau Mengen in diesen Zellen. Diese Ergebnisse zeigen eine bedeutende Rolle für mitchondriale Veränderungen in einer frühen Phase der Alzheimer Erkrankung.

Table of Contents

Abbreviations

ABAD. *Alcohol dehydrogenase*

AD. *Alzheimer's disease*

AMPAR. *α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor*

AP. *Action potentials*

APP. *Amyloid Precursor Protein*

Ascl1. *Achaete-scute homolog 1*

ATP. *Adenosine triphosphate*

Aβ. *Amyloid- β*

BBB. *Blood–brain barrier*

BMP. *Bone morphogenetic protein*

CamKII. *Ca2+/Calmodulin-dependent kinase II*

CNS. *Central nervous system*

CREB. *cAMP response element-binding protei*

CSF. *Cerebrospinal fluid*

CTD. *C-terminal domain*

CTF. *C-Terminal fragment*

ELISA. *Enzyme-linked immunoabsorbant assay*

EpiSC. *Epiblast stem cells*, *Epiblast stem cell*

ER. *Endoplasmic reticulum*

ESC. *Embryonic stem cell*

FAD. *Familial Alzheimer's Disease*

FGF. *Fibroblast growth factor*

GOI. *Gene of interest*

GWAS. *Genome-wide association study*

H^+. *Proton*

Hdac. Histone deacetylase

HDR. *Homology directed repair*

ICM. *Inner cell mass*

iPSC. *Induced pluripotent stem cell*

LOAD. *Late onset alzheimer's disease*

LTD. *Long-term depression*

LTP. *Long-term potentiation*, *Long-term potentiation*

MCI. *Mild cognitive impairment*

mEPSC. *Miniature excitatory postsynaptic potential*

mGlurR. *Metabotropic glutamate receptor*

MMSE. *Mini-Mental State Examination*

mtDNA. *Mitochondrial DNA*

nAcChR. *Nicotinic acetylcholine receptor*

NFT. *Neurofibrillary tangle*

Ngn2. *Neurogenin2*

NHEJ. *Non-homologous end joining*

NMDAR. *N-Methyl-D-aspartate receptor*

NPC. *Neuronal progenitor cell*

PAG. *Pluripotency–associated gene*

PD. *Parkinson's disease*

PET. *Positon emission tomography*

PHF. *Paired helical filament*

PiB. *Pittsburg compound-B*

PTB. *Pyrimidine-tract-binding*

REST. *RE1-silencing transcription factor*

ROS. Reactive oxygen species

SAD. *Sporadic Alzheimer's disease*

SCNT. *somatic cell nuclear transfer*

SNP. *Single-nucleotide polymorphism*

ssODN. *Single-stranded oligonucleotide*

TF. *Transcription factor*

TGF-β. *Transforming growth factor-β*

WHO. *World Health Organization*

Chapter I

Introduction

1.1 Alzheimers Disease

1.1.1 History and Prevalence

Alzheimer's disease (AD) is the most common form of dementia with 50–70 % of all cases. Earliest sings of onset are difficulties to remember recent events and with progression mood swings, loss of orientation, loss of the ability to articulate and more severe memory impairment (Winblad et al., 2016).

In 1906 the physician Alois Alzheimer described the case of Auguste Deter who developed an unusual form of dementia at the age of 51, suffering from memory loss. Deter died a few years later and her brain biopsy showed shrinkage and abnormal deposits in tissue (Alzheimer, 1907). Alzheimer observed a widespread tangle and plaque pathology, which was also described by Fischer in the same year in senile dementia (Fischer, 1907). In 1910 Emil Kraepelin termed this medical condition "Alzheimer's disease". In 1927 Paul Divry, using congo-red stain, identified the composition of plaques to be "amyloid", a term used for aggregates that had properties similar to starch (Divry, 1927). The development of electron microscopy enabled a better analysis of plaques and identified their structure (Kidd, 1963). First protein isolations from AD brains made it possible to get access to tangles (Iqbal et al., 1974) and advances in brain imaging improved dementia prediction (De Leon et al., 1979). On a neuropsychological level the development of the Mini-Mental State Examination (MMSE) enabled to assess the mental status quo of patients (Folstein et al., 1975). In the 80's, first evidence showed reduced brain metabolism and hippocampal atrophy of mild-cognitive impairment (MCI) have a predictive value for a later conversion to AD (De Leon et al., 1989). In the same decade amyloid-β (Aβ) was isolated from human brains (Masters et al., 1985) and the peptide identified to originate from the amyloid precursor protein (APP) (Kang et al., 1987). Together with a study showing microtubule-associated protein tau as integral part of neurofibrillary tangles (NFTs) (Quinlan et al., 1986), which was found to be abnormally phosphorylated in disease (Grundke-Iqbal et al., 1986), the two pathological hallmark proteins of AD were identified. Evidence that Aβ is toxic to neurons and might be responsible for brain atrophy (Yankner et al., 1990), as well as correlation between synapse loss and memory decline were found in subsequent studies (Terry et al., 1991). In the last 30 years remarkable progress, such as a deeper understanding of APP processing, the discovery of familial AD (FAD) mutations and the mapping of late onset AD (LOAD) mutations by genome-wide association studies (GWAS) have been made (further information to these points below). 47 million people suffered from dementia in 2015 with an estimated increase to 131 million by 2050 (Prince et al., 2015). The World Health Organization (WHO) described AD as the fastest growing epidemic in their 2015 report (WHO, 2015). About 5% of the AD cases represent FAD, with an onset before 65, with the remaining 95% being LOAD. Age is the largest risk factor to develop dementia, accordingly the prevalence doubles every 6.3 years in Western Europe (Prince et al., 2013). In the age group 60 – 64 1.9% have dementia, in the next 5 years, 2.6% and at the age of 70–74, this increases to 4.3%. Approaching older ages also reflects on the prevalence in these age groups. People of 75–79

years have 7.3% dementia cases amongst them and in 80–84 year olds this increases further to 12.4%. One fifth of people at the age 85–89 suffer from dementia and close to 40% of people older than 90 years. For Western Europe this translates to 10.46 million out of 176.61 million people (5.9%) over the age of 60 suffering from dementia in 2015. Worldwide 897 million people are over 60, with 5.2% affected by dementia (Prince et al., 2015). In Switzerland out of 1.38 million people above the age of 64 around 110,000 persons were affected in 2011, the estimations for the year 2016 were 127,000 dementia patients (Pfeil et al., 2012). Especially due to longer life expectancy the number of affected people is expected to rise. Most of the risk of developing AD is due to genetic determinants and thus not modifiable (discussed in detail below), whereas estimates predict up to 35% of the AD cases could be preventable (Livingston et al., 2017). These modifiable risk factors include a low level of education (8%), mid-life hearing loss (9%), hypertension (2%) and obesity (1%), and late-life risk factors include smoking (5%), depression (4%), physical inactivity (3%), social isolation (2%) and diabetes (1%).

1.1.2 Clinical Appearance and Assessment

AD builds up over the course a decades, yet clinical symptoms mostly start to show after the age of 65. Late-stage AD patients are unable to perform easiest every-day routines and need constant care. They lose awareness of their surroundings and are unable to recall events from their past. Further, physical abilities deteriorate and patients become vulnerable to infections (Winblad et al., 2016). Newly diagnosed patients are in a very mild stage for a few months, before progressing to an around 2 year-long mild dementia phase. Patients convert to moderate dementia, which lasts 1 – 2 years, and suffer from severe AD for a year (Rizzuto et al., 2012). Yet, progression is highly variable with about 50% of the cases reaching the most severe stage within 3 years, according to another study (Brodaty et al., 2012). Mean survival of affected patients is about 3 – 9 years after diagnosis, but survival can also exceed 15 years (Todd et al., 2013). The average years a person would have lived longer without dementia is 4.9 in the 75 – 84 age group and 2.7 years above the age of 85 (Winblad et al., 2016). Earliest changes in AD affect the region of the medial temporal lobe, such as hippocampus and entorhinal cortex, and disrupt episodic memory (Braak and Braak, 1991). Of note is that amyloid deposition, which occurs prior to symptoms, is rather observed in regions of the default mode network (LaViolette et al., 2009). In patients with mild dementia semantic memory is affected. This affected the naming of objects, verbal expression and general knowledge and reflected the spread of pathology to parietal, temporal, and frontal lobe (Rogers and Friedman, 2008). Other apparent symptoms in MCI to early AD stage are difficulties to understand concepts and solve problems, which was linked to NFT pathology in the prefrontal cortex (Back-Madruga et al., 2004).

Clinical assessment of patients differentiates between preclinical AD, MCI due to AD and dementia due to AD. These diagnostic criteria are based on biomarkers and neuropsychological testing (Albert

et al., 2011; Knopman et al., 2011; Sperling et al., 2011). Biomarker testing can detect alterations in the brain preceding cognitive and behavioral symptoms. Two principal biomarkers can be distinguished, brain imaging and biochemical analysis of cerebrospinal fluid (CSF). Visualization of brain amyloid load is done by positron emission tomography (PET) of Pittsburg compound-B (PiB), an ^{11}C-labelled amyloid binding dye. PiB is retained in regions with high amyloid load and normalized to cerebellum as a reference region (Klunk et al., 2004). CSF samples can be analyzed for different APP processing products, such as Aβ40, Aβ42 and Aβ38, as well as for tau and phosphorylated tau levels (Welge et al., 2009). Enzyme-linked immunoabsorbant assays (ELISA) is used and has been best established for Aβ42 (Motter et al., 1995) and later for (phosphorylated-) tau (Itoh et al., 2001). Aβ42 levels are decreased in CSF samples of AD patients, whereas tau levels increase compared to controls (Tapiola et al., 2009). For neuropsychological testing a range of tests have been developed, such as the AD Assessment Scale-Cognitive (Mohs and Cohen, 1988), MMSE, Instrumental Activities of Daily Living Scale (Katz, 1983), Consortium to Establish a Registry for Alzheimer's Disease (CERAD) test (Hirai et al., 2001), or the Cambridge Neuropsychological Test Automated Battery (Robbins et al., 1994). These tests aim to determine the level of cognitive impairment by studying the subjects short- and long-term memory, ability to solve problems, attention, concentration, and reasoning.

An oldest old study (>90 years old) that showed that 61% of the cohort had signs of dementia on autopsy, but 85% were diagnosed with AD and further, 22 % of the demented patients, had not enough pathology to account for their cognitive loss (Corrada et al., 2012). Therefore, a rigorous and strict assessment of both, biomarkers and neuropsychological testing, is needed to allow prediction of the severity of AD in a patient.

1.1.3 Cellular AD

Changes on a biochemical and cellular level lead to brain alterations resulting in brain atrophy and memory loss. These alterations precede clinical symptoms of AD, changes in CSF Aβ42 levels can be measured 25 years before onset, amyloid deposition in the brain 15 years before, as can be increased tau and phosphorylated tau levels in CSF (Bateman et al., 2012). It is not clearly understood what drives these changes in the asymptomatic phase of AD. All cell types of the brain are known to change in the course the disease, neurons, astrocytes, oligodendrocytes, microglia and endothelial cells (de Strooper and Karran, 2016). Central to the disease are neurons, which are the main source of Aβ and tau, and which represent the most vulnerable cell type. It is thought that initial changes in Aβ and tau, such as aggregation and conformational changes by phosphorylation, lead to defective clearance and thus to disease progression, as indicated by the risk factors APOE4, PICALM and CLU (Bell et al., 2012; Tarasoff-Conway et al., 2015; Verghese et al., 2013; Zhao et al., 2015). Clearance from the brain is done via the blood–brain barrier (BBB), where astrocytes form a functional exchange unit

with pericytes and endothelial cells, enclosing the lumen of a capillary. Furthermore, astrocytes are central to ApoE and cholesterol production (Pfrieger and Ungerer, 2011) and also have an important role in synaptogenesis and synapse maintenance (Thompson et al., 2016). Another component which was found to be misregulated in AD is the immune system and in particular microglia, highlighted by TREM2 risk variants (Sims et al., 2017), a protein essential in microglia function and Aβ phagocytosis (Yeh et al., 2016). Further studies observed that microglia are also involved in synapse loss in AD (Hong et al., 2016; Spangenberg et al., 2016) and increased expression of anti-inflammatory interleukin 10 led to lower Aβ plaque load (Chakrabarty et al., 2015). Together these findings show the complex interplay between different cell types and their pathological changes in AD.

1.1.4 APP processing and amyloid-β

The APP protein is encoded on chromosome 21 and has three major isoforms, generated by alternative splicing. APP is a transmembrane protein with a single transmembrane domain. Neurons express APP695, whereas APP751 and APP770 (named according to their length in amino acids) are the predominant forms in other tissues (Kang et al., 1987). APP is found to be enriched at active zones (Wilhelm et al., 2014) and intracellular compartments (DeBoer et al., 2014). The biological function of APP is addressed by various studies, which find the protein to be involved in diverse processes, including cell survival, synaptic stability, and plasticity (Müller et al., 2017).

Proteolytic processing of APP generates different fragments (Figure 1.1.). We discriminate between the non-amyloidogenic pathway and the amyloidogenic pathway, generating Aβ. In non-amyloidogenic processing APP is cleaved by α-secretase (mainly ADAM10, but also ADAM9 and 17). This releases the ectodomain sAPPα (AA 1 – 612) into the extracellular space. The resulting membrane bound fragment α C-terminal fragment (αCTF or C83) is further cleaved by the γ-secretase complex (presinilin1 and 2 with nicastrin, presinin enhancer 2, anterior pharynx-defective 1 and 2). This releases the p3 fragment and the APP intracellular domain (AICD). In the amyloidogenic pathway APP is processed by β-secretases (BACE1 and BACE2) generating the soluble sAPPβ (AA 1 – 596) and βCTF (C99). Subsequent γ-secretase cleavage releases Aβ and AICD. The γ-secretase complex can generate different lengths of Aβ peptides. These are parted in a major, Aβ40 generating, and a minor, Aβ42 generating, product line. In the Aβ40 line the first Aβ peptide is Aβ49 (ε-cleavage site of γ-secretase), subsequent trimming by 3 AAs generates Aβ46 (δ-cleavage), Aβ43, Aβ40 and Aβ37, with Aβ40 being the most abundant form. The Aβ42 production line starts with Aβ48 and generates Aβ45, Aβ42, and Aβ38. In these competing pathways, 10-fold more Aβ40 than Aβ42 is generated (De Strooper, 2010). APP is most abundant in endoplasmic reticulum (ER) and Golgi apparatus and α-secretase cleaves at physiological pH, whereas β-secretase processing requires a lower pH, as found in early endosomes (Haass et al., 2012; Rajendran et al., 2006). Thus, it is accepted that both processing pathways are active in cellular distinct compartments.

Canonical APP processing is represented by α-, β-, and γ-secretase cleavage, yet also other, non-canonical, processing mechanisms have been identified. Δ-secretase has two cleavage sites within APP. At AA 373 and AA 585, thus proteolytic processing creates sAPPδ (1 – 585) and/or sAPPδ (1 – 384) and sAPPδ (384 – 585). The δCTF can be further processed by β-secretase and led to Aβ production (Zhang et al., 2015). The metalloprotease MT5-MPP contributes to η-processing of APP. It cleaves APP after AA 504, creating sAPPη and ηCTF. Latter can be cleaved by ADAM10 or BACE1, leading to αCTF and Aη-α or βCTF and Aη-β, respectively. Aη-α was found to affect long-term potentiation (LTP) in hippocampal slices (Willem et al., 2015). Moreover, meprin-β has been shown to cleave APP (Bien et al., 2012). Non-canonical processing pathways of APP are not researched in depth. Further research is required to understand their biological relevance, as well as their role in disease.

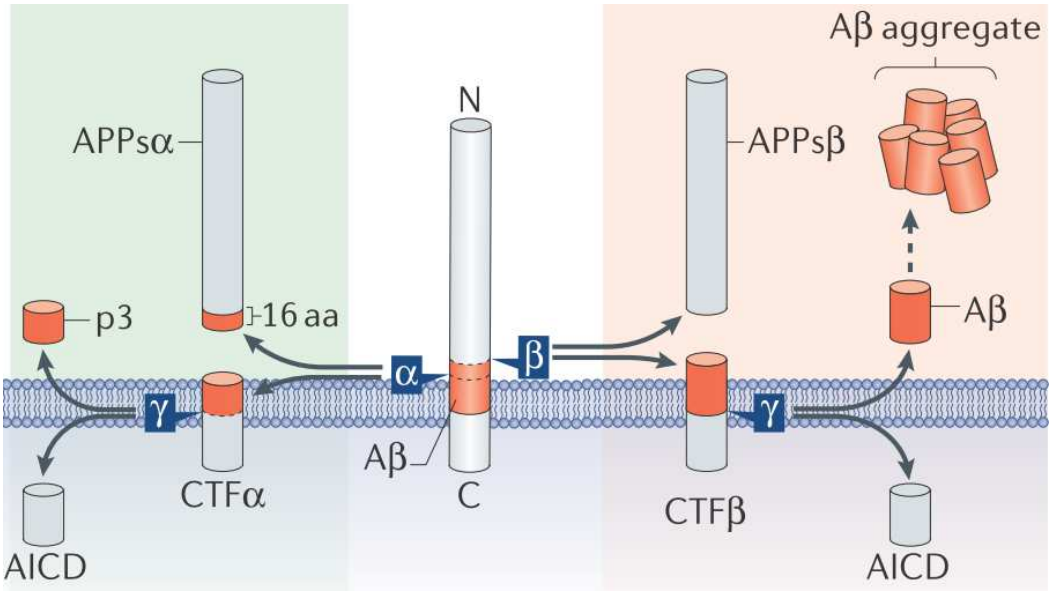

Figure 1.1 Proteolytic processing of APP. APP is cleaved by α- or β-secretase to generate sAPPα and αCTF in the non-amyloidogenic pathway, or sAPPβ and βCTF in the amyloidogenic pathway. Subsequent processing of αCTF by γ-secretase releases the p3 peptide and AICD, whereas γ-secretase cleavage of βCTF generates the toxic Aβ peptide and AICD. Not that both processing pathways occur in distinct cellular compartments. Image modified from (Müller et al., 2017).

1.1.5 Microtubule associated protein tau

The microtubule associated protein tau is encoded in the *MAPT* gene on chromosome 17 (Goedert et al., 1988). It is expressed in neurons and at low levels in glia (LoPresti et al., 1995). Alternative splicing of *MAPT* pre-mRNA leads to the generation of six different isoforms. These differ in Exon 2, 3 or 10. The first two lead to an insert near the N-terminus, with either both exons spliced out (0N), one exon spliced out (1N) or both exons retained (2N). Exon 10 is located in the C-terminal repeat domain, tau with exon 10 has four repeats (4R) and without three repeats (3R). Fetal brain expresses

only 0N3R tau with a shift in adult brains to equal levels of 3R and 4R tau. More than half of the adult tau is 1N, 37% 0N, and 9% 2N (Goedert and Jakes, 1990). With the additional repeat region, 4R tau has been shown to more efficiently promote microtubule assembly than 3R tau (Goedert and Jakes, 1990). Tau distributes throughout the cell body and neurites in young neurons and upon axon polarization localizes to axons (Papasozomenos and Binder, 1987). In axons tau is involved in cytoskeleton reorganization by binding to microtubules, which stabilizes them and promotes their assembly (Mandelkow and Mandelkow, 2012). Different mechanisms for axonal transport regulation by tau have been proposed. Tau competes with the motor proteins dynein and kinesin for microtubule binding and thereby reduces the run length and frequency of transport *in vitro* (Dixit et al., 2008). Further, it was shown, that tau reduces the number of motor protein engaged in transport *in vitro* (Vershinin et al., 2006), yet *in vivo* tau deletion or overexpression did not affect transport (Yuan et al., 2008). Tau is released into the extracellular space depending on synaptic activity (Pooler et al., 2013). 85 phosphorylation sites are present in 2N4R tau, of which 45 have been confirmed (Hanger et al., 2009). On average adult tau carries two phosphates, whereas in AD tau is phosphorylated on approximately 8 sites (Köpke et al., 1993). Tau phosphorylation has been shown to play a crucial role in tau function and phosphorylation in the repeat domain reduced the affinity to microtubules (Hanger et al., 2009). Hyperphosphorylation induced tau missorting and thereby led to spine loss (Thies and Mandelkow, 2007), whereas contradicting studies show that GSK-3β mediated tau phosphorylation did not lead to spine loss (Tackenberg and Brandt, 2009). Tau can aggregate into paired helical filaments (PHFs) and NFTs and abnormally phosphorylated tau has been found in AD brains. Isolates from brains were able to self-assemble into PHFs (Alonso et al., 2001). Yet, tau aggregation *in vitro* did not require phosphorylation (Goedert et al., 1996) and cofactors which could be required for aggregation might be involved in this process. New venues for tau-mediated neurodegeneration are findings of tau spreading in a prion-like manner. Tau aggregates (trimers or larger (Mirbaha et al., 2015)) were found to be able to exist in different conformations (Sanders et al., 2014) and the propagation of these misfolded strains was enhanced on neuronal activity (Wu et al., 2016). The mechanism of tau transmission is thought to be trans-synaptic spreading, leaking from damaged neurons, or by exocytosis and is still subject of investigation. In AD, tau pathology starts in the entorhinal cortex, progresses to the hippocampus and later to the cortex (Braak and Braak, 1991). Prion-like spreading could be an explanation for the progression of tau pathology in AD but requires further investigation.

1.1.6 Amyloid-β toxicity

The amyloid cascade hypothesis proposes that gradual accumulation of Aβ initiates the detrimental effects of AD (Hardy and Higgins, 1992). Aβ has been identified as a major source of toxicity in AD (Mucke and Selkoe, 2012). It exists in different aggregation forms, from monomers to oligomers to

fibrils, which ultimately form plaques. These aggregates are thought to differentially contribute to disease progression. It has been shown, that the plaque load in AD patient brains does not correlate with disease severity (Perrin et al., 2009), whereas Aβ oligomers are thought to substantially contribute to toxicity and neuronal cell death (Benilova et al., 2012). As described above, APP processing generates different Aβ fragments, with the most abundant being Aβ40 and Aβ42. These fragments displayed different aggregation propensities (Yan and Wang, 2006). Aβ oligomers were found to interact with a range of receptors and have been shown to be a potent inhibitor of LTP (Walsh et al., 2002), led to spine loss (Shankar et al., 2007) and disrupted cognitive function (Cleary et al., 2004). It has been observed that antibodies against the N-methyl-D-aspartate receptor (NMDAR) prevent Aβ binding to spines (de Felice et al., 2007) and pharmacological inhibition of NMDARs abolished spine loss (Tackenberg et al., 2013). Further, calcineurin and caspase-3 activation have been shown to mediate NMDAR-dependent spine loss (Tackenberg and Brandt, 2009; Tackenberg et al., 2013). Aβ oligomer-mediated LTP reduction has been shown to depend on metabotropic glutamate receptors (mGlurRs) (Wang et al., 2004)(Palop and Mucke, 2010) and upregulation of the nicotinic acetylcholine receptor (nAcChR) by Aβ induced synaptotoxicity (Dineley et al., 2001). Other effects of Aβ-mediated dysfunction were increased tau phosphorylation upon Aβ oligomer treatment and tau missorting into dendrites (Zempel et al., 2010). Moreover, intracellular Aβ led to increased mitochondrial stress (Kondo et al., 2013) and treatment of primary neuronal cultures with Aβ caused neuronal death (Ahmed et al., 2010). Taken together, toxicity of the Aβ peptide has been shown to affect proper neuronal function in various ways and it is likely that a combination of effects leads to neuronal death.

1.1.7 Familial AD

About 5% of AD cases are early-onset AD, and half of those carry an autosomal dominant mutation. A genetic hallmark of FAD is a near complete penetrance of a genetic mutation in APP, PSEN1 or PSEN2 (Bekris et al., 2010), leading to an onset of the disease before the age of 65 (Winblad et al., 2016). So far, over 20 mutations in APP and over 200 in PSEN1/2 have been identified (Alzforum). APP mutations have been mainly identified around the β- and γ-cleavage site and in the middle of the Aβ peptide. Depending on their localization, assumptions can be made on their effect. Mutations in the middle of Aβ lead to increased aggregation propensity of the peptide, such as seen in the E693Δ mutation. Mutations around the β-secretase site increase the amyloidogenic processing and lead to a higher production of total Aβ. Mutations in vicinity of the γ-secretase processing site of APP change the ratios at which Aβ40 and 42 are generated, with an increase of the more toxic Aβ42 (Ridge et al., 2013). It has been shown that PSEN mutations lead to a destabilization of the APP – PSEN interaction, making an earlier release of the substrate more likely, which increases the amount of Aβ42 generation (Szaruga et al., 2017). It has to be mentioned that mutations in those genes are not always

pathogenic but can also be protective variants, as identified in an cohort from Iceland (Jonsson et al., 2012). As only half of the early-onset patients carry mutations in APP, PSEN1 or 2, some genomic variants are still to be discovered and are likely to have a very low frequency in the population.

1.1.8 Sporadic AD

About 65 % of the total risk to develop AD is determined by genetics, with the remaining 35% being environmental factors (Livingston et al., 2017). With 95% of the AD cases being sporadic AD (SAD), it represents the majority of all cases. While FAD mutations have a high penetrance, risk factors for SAD increase the chances to suffer from AD at some point. Risk altering SAD mutations have been identified in GWAS studies. Hereby, single-nucleotide polymorphisms (SNPs), which are inherited in linkage disequilibrium, are sequenced in a cohort of SAD patients and controls. The frequency of a genetic variant is correlated to their phenotype (Lambert et al., 2013) and thereby risk loci are identified. Newer studies used additional approaches, such as whole-exome microarrays (Sims et al., 2017) or whole-exome sequencing (Guerreiro et al., 2013) to identify novel risk variants. The variants conferring the highest risk were found in APOEε4 (odds ratio 3.78), TREM2 (5.05), SORL1 (1.3), BIN1 (1.22), CR1 (1.18), CLU (1.16), PICALM (1.15), and ABCA7 (1.15). Over 20 risk factors have been identified so far (Van Cauwenberghe et al., 2015). A second factor to take into consideration is the allele frequency within the population. Here, only APOEε4 had a high frequency (30.8%) (Van Cauwenberghe et al., 2015), whereas all other variants were present in fewer than 10% of the cohorts. TREM2 variant, though having a high odds ratio, was present in less than 1% of the population. APOE a lipid-binding protein mainly produced in astrocytes has three different isoforms, ε2, ε3, and ε4. While APOE 3/3 is the most common genotype and does not affect the AD risk, the presence of one or two copies of APOE 4 (APOE 3/4 or APOE 4/4) have been shown to increase the risk by tree or twelve fold, respectively (Michaelson, 2014). In contrast, APOE 2 was found to reduce the risk for AD (West et al., 1994). Genetic testing in early-onset AD can be highly predictive, given the high penetrance. For SAD, genetic variants confer a susceptibility to AD, but the individual genetic diagnosis cannot reliably predict the diseases outcome, as even two APOEε4 alleles are neither sufficient nor necessary to lead to AD.

1.2 Induced pluripotent stem cells

1.2.1 Definition and development of induced pluripotent stem cells

The technology of induced pluripotent stem cells (iPSCs) is based on scientific advances and discoveries in the past. Stem cells are defined by two criteria, potency and self-renewal. Pluripotency describes the developmental potential of iPSCs to differentiate into any specialized cell of the body. In

contrast, adult stem cells, such as neural stem cells, are multipotent and thus show a degree of lineage commitment. The term “induced” can be attributed to the fact that iPSCs originate from a somatic cell that has been reprogrammed to stem cell state by expression of ectopic transcription factors.

The development of iPSC technology was awarded the 2012 Nobel Prize in Physiology and Medicine to John B. Gurdon and Shinya Yamanaka. In 1962 Gurdon and colleagues transferred the nucleus from intestinal cells of adult frogs into unfertilized eggs and observed that these developed into tadpoles. This method was termed somatic cell nuclear transfer (SCNT) (Gurdon, 1962). It showed that the nucleus contained all the necessary information to form an entire organism. A series of experiments led to the discovery of master transcriptional regulators. Treatment of fibroblasts with 5-azacytidine, a blocker of methyltransferase, converted those to myogenic cells, adipocytes, and chondrocytes by demethylation of genes (Taylor and Jones, 1979). Transfection of DNA from those converted cells could induce myoblast fate (Lassar et al., 1986) and subsequently, MyoD overexpression was sufficient for conversion of fibroblasts to myocytes (Davis et al., 1987). Research on embryonic stem cells (ESCs) revealed how to culture isolated mouse ESCs (mESCs) (Evans and Kaufman, 1981) and later to long-term maintain pluripotency. Essential were the discovery of mESCs dependence on leukemia inhibitory factor (LIF) (Smith et al., 1988) and the generation of human ESCs (hESCs) (Thomson et al., 1998). In 2006, these three lines of research converged and led to the identification of transcriptional regulators of stem cell fate that are capable to reprogram somatic cells and maintain this state *in vitro,* with the initial reprogramming factors Oct4 (also called Oct3/4 or Pou5f1), Sox2, Klf4, and c-Myc (termed OSKM) (Takahashi and Yamanaka, 2006). The following year, the groups of Yamanaka and Thomson identified the conditions for the derivation of human iPSCs from fibroblasts, using the above mentioned transcription factors (TFs) (Takahashi et al., 2007) or Oct4, Sox2, Nanog, and Lin28 (Yu et al., 2007). Candidate genes of pluripotency-associated transcription factors were identified in prior studies that showed Oct4 and Sox2 as core transcription factors of pluripotency (Avilion et al., 2003; Looijenga et al., 2003). Furthermore, Nanog overexpression enabled mESCs to self-renew in the absence of LIF (Mitsui et al., 2003). This further revealed that KLF4 is a downstream target of Stat3, which is activated by LIF (Lin et al., 2005; Niwa et al., 2009). Myc was found to be necessary for mESC maintenance (Cartwright et al., 2005) and thus added to the candidate list. Together with additional factors, iPSC induction was tested in a reporter cell line and lead to the identification of OKSM (Takahashi and Yamanaka, 2016). This discovery opened new avenues of research. Differentiated somatic cell derived from iPSCs are nowadays used for drug screening, disease modeling or patient-specific cell therapy.

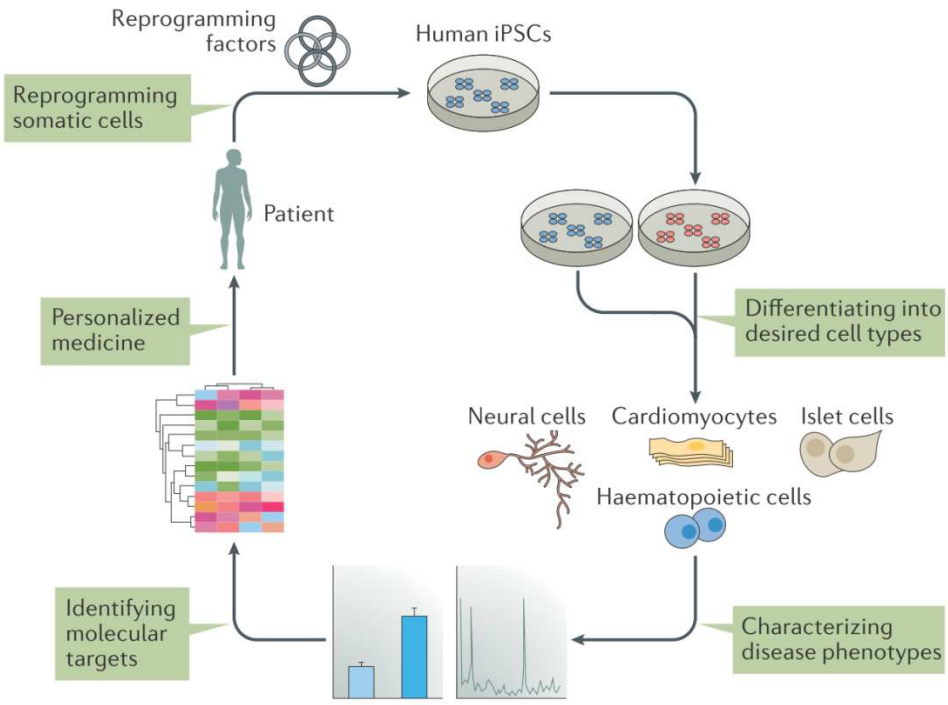

Figure 1.2 Disease modeling with human iPSCs. Somatic cells are reprogrammed to iPSC state. These can be genetically modified by Crispr-Cas9 to create isogenic lines, or patients and control cells are differentiated to a cell type of interest. Subsequent identification and characterization of phenotypes allow examination of molecular pathological mechanisms and enable discovery of new drugs and personalized medicine. Modified from (Shi et al., 2016).

1.2.2 Reprogramming of somatic cells to pluripotency

Reprogramming induces widespread changes in chromatin organization and transcription. Expression of reprogramming factors with the subsequent conversion to iPSCs can be divided into two different phases, early and late reprogramming. During these phases OKSM occupy different genomic locations to activate the endogenous core pluripotency network while repressing somatic cell fates. Expression of OSKM leads to downregulation of the Thy1, a fibroblast marker, and was followed upregulation of SSEA1 in mouse and Tra-1-60 in human cells (Anderssen et al., 2012; Chan et al., 2009). These cells subsequently silence mesenchymal genes and undergo mesenchymal-epithelial transition (MET), observed by the upregulation of markers such as Chd1 and Epcam. This process is promoted by transforming growth factor-β (TGF-β) inhibition and bone morphogenetic protein (BMP) signaling pathway activation and leads to a higher proliferative state (Li et al., 2010a; Zunder et al., 2015). Subsequently, transcriptional regulators Nr0b1 and Etv5 are activated, followed by pluripotency factors Sox2, Rex1 and Dppa2 (Lujan et al., 2015). On a genomic level in the early phase of reprogramming, OSK bind to regions of open chromatin, representing regions of genes for somatic cell identity, which they normally do not occupy in established pluripotency. OSK preferentially bind to enhancers, whereas Myc preferentially binds promotors (Kim et al., 2008; Soufi et al., 2012). Binding of OSK redistributes somatic TFs concomitant with histone deacetylase 1 (Hdac1)

recruitment to OSK bound regions, which leads to inactivation of somatic genes (Chronis et al., 2017). OSKM also bind enhancers and promoters of pluripotency–associated genes (PAGs) in the early reprogramming phase, yet their activation is repressed by histone methylation, making DNA less accessible for binding but not impossible. It was observed that these early targets have a high density of OSK motifs (Chronis et al., 2017). Occupancy of OSKM as pioneering factors for chromatin remodeling in the early phase was observed to lead to initial expression of early pluripotency genes (Soufi et al., 2012). These changes were thought to be the major roadblock in reprogramming, yielding in low efficiencies. The second phase of reprogramming was described as a more hierarchical process, in comparison to the stochastic early phase (Buganim et al., 2012). The upregulation of Oct4 together with Sall1 and Sall4 was found to activate late reprogramming genes such as Nanog , Essrb and Klf2 (O'Malley et al., 2013). Activation of the endogenous PAGs lead to feed-forward responses, self-enhancing the pluripotent circuit (Takahashi and Yamanaka, 2016). Myc was found to be dispensable for reprogramming but increases the efficiency by increasing the proliferative state (Wernig et al., 2008). In line, Myc depletion in mESCs was shown to induce a state similar to diapause, leading to proliferative arrest without affecting pluripotency (Scognamiglio et al., 2016).

The efficiency of reprogramming is generally low, with about 0.01–1%, depending on the method used (Schlaeger et al., 2014). It was reported that the stoichiometry of OSKM influenced the generation of iPSCs, with high Oct4 and low Sox2 levels being most efficient (Carey et al., 2011; Papapetrou et al., 2009). As reprogramming is a highly complex process, efficiency can be enhanced in different ways. To mention a few, it was shown that addition of other PAGs, such as Lin28, enhanced generation as well as knock-down of p53, a protein involved in DNA damage recognition at the G1/S cell cycle check point (Okita et al., 2011; Rasmussen et al., 2014). Hypoxia during reprogramming was found to lead higher number of cells (Yoshida et al., 2009). Further, addition ascorbic acid, inhibition of GSK3-β (Bar-Nur et al., 2014) and activation of Wnt signaling (Marson et al., 2008) improved iPSC yield. Moreover, sodium butyrate, a Hdac inhibitor, has been shown to enhance the reprogramming process (Mali et al., 2010). In line, knockdown of Mbd3, which is part of the Nucleosome remodeling Deacetylase (NurD) complex, led to a near 100% efficiency (Rais et al., 2013), however contracting evidence has been found, stating that NurD has an important function in acquisition of pluripotency (Santos et al., 2014).

Since the development of iPSCs, different reprogramming methods have been established. These can be divided into integrating and non-integrating techniques. Retro-/lentiviral delivery of OKSM is the most commonly used integrating method (Carey et al., 2008; Takahashi and Yamanaka, 2006), with the drawback that genes might be disrupted, transgenes could activate neighboring genes, and reprogramming factors could be reactivated (Takahashi and Yamanaka, 2016). For non-integrating reprogramming, episomal vectors (Okita et al., 2011; Yu et al., 2009), sendai-virus (Fusaki et al., 2009), mRNA transfection (Warren et al., 2010) and less commonly, piggyback mediated OSKM transposition (Woltjen et al., 2009) are used. Comparison of the prior three and lentivirus mediated

reprogramming showed highest efficiency for mRNA based reprogramming and least for episomal plasmids. However, the success rate of mRNA based reprogramming was only 20% compared to about 90% for other methods. Aneuploidy rate, an abnormal karyotype due to reprogramming, was detected in 11.5% of iPSCs after reprogramming with episomal vectors, 4.5% for Lenti- and Sendaivirus and 2.3% for mRNA (Schlaeger et al., 2014). Reprogrammed cells should in theory give rise to iPSCs that are not distinguishable to ESCs in their epigenetic profile. Yet it has been found that residual methylation patterns of the cell type of origin affected the differentiation potential of iPSCs (Kim et al., 2010). All of the initial reported reprogramming factors are exchangeable (Theunissen and Jaenisch, 2014). Use of a different set of factors, Sall4, Nanog, Esrrb and Lin28, enabled generation higher quality miPSCs, whereas this has not been reported for hiPSCs (Buganim et al., 2014). Further research showed that reprogramming led to aberrant silencing of the *Dlk1-Dio3* locus, which has an important role in development (Stadtfeld et al., 2010; da Rocha et al., 2008). Reprogramming in the presence of ascorbic acid mitigated methylation of *Dlk1-Dio3* and generated developmentally more potent miPSCs (Stadtfeld et al., 2012). Further effects of culture conditions on iPSCs are described below (see 1.2.3).

After reprogramming iPSCs have to be tested for different criteria to confirm acquisition of pluripotency. The stringency depends on the subsequent use of these cells and indicates their grade of pluripotency (De Los Angeles et al., 2015). *In vitro* assays include examination for an ESC-like morphology, expression of PAGs, and differentiation to cells of the three germ layers (Martí et al., 2013). Using markers for genes activated late in reprogramming are a good predictor to distinguish between partially reprogrammed cells and fully reprogrammed cells (O'Malley et al., 2013). Teratoma formation is used to assess the spontaneous *in vivo* differentiation potential, by staining teratomas for markers of the different germ layers. The most stringent test ethically allowed for human iPSCs is the evaluation of interspecies chimerism, hiPSCs are injected in d10 mouse embryos and contribution of human cells is assessed after birth. This tests whether iPSCs can participate in normal development (Nagy et al., 1990). Further examinations, only carried out with miPSCs, include 2n complementation and 4n complementation assays, latter assessing the potential of a SC to develop an entire organism (Nagy et al., 1993).

1.2.3 Maintenance of iPSCs

Culturing conditions have a major impact on the maintenance of pluripotency and properties of iPSCs. Human ESCs were isolated from the inner cell mass (ICM) of the pre-implantation blastocyst (Thomson et al., 1998) which exists at day 6–7 of human embryonic development. Attainment of pluripotency was found to depend on fibroblast growth factor 2 (FGF2) and TGF-β signaling. Prior activates MEK and ERK signaling pathway and leads to expression of pluripotency genes (Li et al., 2007). Latter leads to phosphorylation of SMAD2/3 and subsequent upregulation of Nanog expression

(Bertero et al., 2015). Culturing of hESCs and hiPSCs was done on γ-irradiated mouse embryonic fibroblasts (MEFs), in feeder-dependent culturing (Takahashi et al., 2007), yet for more defined culture conditions and clinical use, xeno-free methods had to be developed. Feeder-independent culture on matrigel, vitronectin (Chen et al., 2011) or laminin521 (Rodin et al., 2014) has been establish, with the first media for feeder-independent iPSCs established in 2009 (Sun et al., 2009) and later a minimal medium to sustain pluripotency (Chen et al., 2011).

In the above mentioned culture conditions, hESCs and hiPSCs resemble mouse epiblast stem cells (EpiSCs), which are isolated from post implantation embryo at day 6–7.5 of mouse development. Their maintenance is dependent on FGF2 and TGF-β, however maintenance of mESCs, isolated from the ICM of the pre-implantation blastocyst, is dependent on LIF signaling (Tesar et al., 2007). Generation of miPSCs in LIF medium (LIF with GSK3β and MEK1/2 inhibitor, called 2i/LIF medium) generates ES-like miPSCs, whereas use of EpiSC medium generates epiblast SC (EpiSC)-like miPSCs (Hanna et al., 2009). Thus, the culturing condition defines the type of created stem cell. EpiSCs are also called primed SCs, as their developmental potency is limited to the three germ layers, and do not have a high contribution to an organism if injected into the blastocyst. In comparison, naïve SCs, which are LIF-dependent, have a high chimeric contribution and should be capable to create an entire organism from a single cell SC, which can be tested by the 4n complementation assay (Theunissen et al., 2016). Other differences between primed and naïve cells are high levels of Histone 3 lysine 27 trimethylation (H3K27m3) on developmental regulators, a mark of inactive genes, in primed SCs. Further naïve mESCs/iPSCs show global DNA hypomethylation and no X chromosome inactivation (Gafni et al., 2013). Moreover naïve cells display a higher expression of pluripotency markers and maintenance of pluripotency is not dependent on expression of epigenetic repressors, such as DNMT1 (Geula et al., 2015; Liao et al., 2015). It was found that human ESCs and iPSCs do not have the same dependence on LIF as mouse ESCs and iPSCs and display the more restricted differentiation potential of primed EpiSCs (Weinberger et al., 2016). Human iPSCs have genetic and epigenetic differences between lines and within clones that have been shown to affect their differentiation potential (Kim et al., 2010; 2011; Kumar et al., 2014; Panopoulos et al., 2017). Derivation of human naïve ESCs and iPSCs could provide a useful tool to overcome this limitation and progress has been made towards this end. Adaptation of culture conditions led to the establishment of different human pluripotent ground states, which were able to recapitulate features of naïve mESCs (Chan et al., 2013; Gafni et al., 2013; Takashima et al., 2014; Theunissen et al., 2016; 2014; Ware et al., 2014). Recently, a publication showed a novel culturing condition, which allowed the generation of a mouse from a single miPSC in the 4n complementation assay, showed up to 1% contribution of human iPSCs in interspecies chimerism and contributed to ICM and trophectoderm, an indicator of totipotency (Yang et al., 2017b). Culture conditions of human and mouse iPSCs are still subject of extensive research and refinement of those will allow for higher stability and quality of iPSCs, which might also affect the quality of iPSC-derived cells.

1.2.4 Differentiation of iPSCs to neuronal cells

The availability of human material for research is limited, especially in regard to cells of the nervous system. This has changed with the discovery of iPSCs, which are capable to differentiate into any cell type of the body and give a source of infinite cell supply. The generated cells provide a resource to investigate mechanisms of development and disease. The specific genetic background of the donor will allow capturing sporadic and genetically determined disease traits (Brennand et al., 2011; Kondo et al., 2013; Mertens et al., 2015b). Differentiation of iPSCs often relies on the timed use of morphogens and mitogens that recapitulate human development with spatial and temporal clues (Chambers et al., 2009). Derivation of various mesodermal, endodermal, ectodermal, and germ cells has been accomplished by these means (Williams et al., 2012). Other methods use TF overexpression to induce cell fate, starting with somatic cell populations (this process is named transdifferentiation) or iPSCs (Vierbuchen et al., 2010; Zhang et al., 2013). Neuronal differentiation using external cues utilizes iPSC-derived neuronal progenitor cells (NPCs) resembling neural rosette NPCs, normally found in the neural tube, and radial glia NPCs, found in the fetal and adult brain (Elkabetz et al., 2008; Koch et al., 2009; Liour and Yu, 2003). In the neural tube a growth factor gradient leads to generation of neurons with a specific regional identity. BMP is produced in the dorsal part, whereas sonic hedgehog (SHH) is secreted from ventral regions (Fuccillo et al., 2006; McMahon et al., 1998). The anterior-posterior axis is defined by retinoic acid (RA) - FGF gradient (del Corral et al., 2003). Supplementing growth factors according to these gradients will generate neuronal cells with regional identity. By now protocols for a range of neuronal subtypes, such has glutamatergic cortical neurons (Chambers et al., 2009; Eiraku et al., 2008; Elkabetz et al., 2008; Shi et al., 2012), dopaminergic neurons (Cho et al., 2008; Nguyen et al., 2011), motor neurons (Sances et al., 2016), GABAergic neurons (Colasante et al., 2015; Sun et al., 2016), or cell of the enteric nervous system (Fattahi et al., 2016) have been established. Further, protocols for other brain cells such as astrocytes (TCW et al., 2017), oligodendrocytes (Douvaras et al., 2014; Wang et al., 2013) and brain resident microglia (Abud et al., 2017; Muffat et al., 2016) were published. Directed differentiation, the overexpression of a fate-determining TF, has the advantage of higher conversion efficiency and shorter derivation times, yet it is unclear whether the cells skip the initial developmental changes, which are recapitulated in growth factor based approaches. Neuronal cells derived by TF overexpression are termed induced neuronal (iN) cells (Vierbuchen et al., 2010) and starting cell types can be somatic cells, such as fibroblasts, or iPSCs and ESCs. Requirements for the efficient conversion of fibroblasts to iN cells is expression of Achaete-scute homolog 1 (Ascl1) and Neurogenin2 (Ngn2), whereas induction from iPSCs only requires one TF, Ngn2, yet this strongly depends on the cell type of interest that has to be generated. Pioneering TFs for neuronal induction are Acsl1 (Chanda et al., 2014), Ngn2 (Zhang et al., 2013), microRNA-9* or 124 (Sun et al., 2011) and also inhibition of pyrimidine-tract-binding (PTB) protein or RE1-silencing transcription factor (REST) (Xue et al., 2013) were shown to be sufficient to induce neuronal fate. Ascl1 was shown to repress many non-neuronal cell fates and thereby act as a pro-

neuronal TF, which is a unique property in contrast to other TFs which have a direct pro-neuronal function (Mall et al., 2017). microRNA-9* and 124 were found to activate Neurod2, another pro-neuronal TF (Sun et al., 2011). PTB downregulation was found to negatively affect REST levels, by de-repressing a microRNA that targets components of the REST complex (Xue et al., 2013). Other important transcription factors in the establishment of neuronal cell fate, mostly used in combination with one of the prior named ones, are Neurod1, Brn2, Myt1l (Ambasudhan et al., 2011; Vierbuchen et al., 2010).

Neuronal cells derived from iPSC or ESC are in an early stage of development and thus shown no age-signature. Interestingly, transdifferentiation of fibroblasts with Ascl1 and Ngn2 led to iN cells which retained their age-signature (Mertens et al., 2015a). This led to the identification of decreased RanBP17 levels in age. Further efforts to induce an age-phenotype were made by overexpression of Progerin, a protein involved in premature aging in Hutchinson–Gilford progeria syndrome (Miller et al., 2013). Ageing of cells might prove a useful tool for the research of late-onset diseases.

Differentiation of iPSCs has expanded to the field of 3D culturing, as this condition is thought to generate more mature cells with a higher level of cellular identity in a more relevant culturing context, recapitulating certain structures of organs. Cells are either differentiated and cultured in a scaffold or generated as organoids to capture specific morphological structures. Differentiation in scaffolds can be carried out in matrigel or polyethylenglycol hydrogels (Kim et al., 2015; Kothapalli and Kamm, 2013; Shepherd and Parker, 2011). Organoids start differentiation with spheroid cell aggregates of iPSCs called embryoid bodies. Differentiation is guided by mitogen and morphogen treatment to recapitulate human brain development (Lancaster and Knoblich, 2014). These organoids were, for example, used to reveal developmental defects in different diseases (Birey et al., 2017; Qian et al., 2016) or examine astrocyte maturation (Sloan et al., 2017).

Recent advances in iPSC differentiation techniques made it possible to generate neuronal populations with better subtype specificity and short differentiation times to study development and disease relevant mechanisms. One of the remaining challenges is to achieve maturity of iPSC-derived cells, but progress in this direction has already been made (Qi et al., 2017).

1.3 Mitochondria

1.3.1 Function of Mitochondria

Mitochondria are the main producer of ATP in the cell and are implicated in Ca^{2+} buffering. The organelle consists of a double membrane and is found in all eukaryotic organisms, their origin is thought to be the endosymbiosis of a prokaryotic organism. Mitochondria have their own DNA, called mitochondrial DNA (mtDNA), though most of the mitochondrial proteins are encoded in the nucleus. About 1200 proteins are encoded in the nucleus, whereas mtDNA encodes 37 genes, 13 proteins, 22 transfer RNAs and two ribosomal RNAs (Quirós et al., 2016). The existence of an inner and outer

membrane is essential for the generation of adenosine triphosphate (ATP), as it allows the establishment of an electrochemical proton (H^+) gradient between the intermembrane space and the matrix of the inner membrane (Frey and Mannella, 2000). The gradient is maintained by the electron transport chain (ETC) with NADH as electron donor and protons being pumped into the intermembrane space to create a proton gradient and an electrical potential. Prior to oxidative phosphorylation, glucose is processed in the glycolysis and citric acid cycle. In glycolysis glucose is broken down to two molecules of pyruvate, 2 ATP, 2 NADH and 2 H_2O (Alberts et al., 2008). Pyruvate is further processed to acetyl-CoA, CO_2 and NADH. The citric acid cycle utilizes the two generated acetyl-CoA molecules for the production of thee NADH, $FADH_2$, and GTP. The NADH and FADH molecules generated in these processes are used in the ETC (Akram, 2014). The ETC consists of five complexes located in the inner mitochondrial membrane. At complex I NADH is oxidized to $NAD^+ + H^+$ and two electrons pass through the complex with ubiquinone as their final acceptor, which is reduced together with two H^+ from the matrix to ubiquinol (QH_2). During the electron transport, four protons are pumped to the intermembrane space. In complex II succinate is oxidized to fumarate and two H^+. The final acceptor of complex II is ubiquinone (Q) which is converted to QH_2.In complex III, the cytochrome c reductase complex, QH_2 is oxidized and two protons released in the intermembrane space. One electron reduces cytochrome c, whereas the other reduces Q to Q^-. In a second step another QH_2 is oxidized, cytochrome c reduced, H^+ pumped outside and Q^- with two H^+ from the matrix and another electron converted to QH_2. Complex IV of the ETC, cytochrome c oxidase, makes use of the reduced cytochrome c from complex III, these electrons are transferred with four H^+ to O_2, forming H_2O, while four H^+ are pumped across the membrane to the intermembrane space. The ATP synthase, complex V, utilizes the electrochemical gradient that was build up by the ETC to generate ATP, allowing H^+ reflux from the intermembrane space to the matrix. Overall about 30 molecules ATP are generated per molecule glucose by oxidative phosphorylation (Hatefi, 1985; Senior, 1988). Yet, this process also leads to the production of reactive oxygen species (ROS), which can cause lipid peroxidation, protein modification, as well as DNA damage and its aberrant production has been implicated in different diseases (Scheibye-Knudsen et al., 2014). Thus, functionality of mitochondria is essential and tightly regulated. Mitochondria are distributed throughout the cell and form a dynamic network. The integrity of this network is essential for cell functionality and neuronal polarization. Regulators of this network are the major proteins involved in fusion and fission. Mitofusin 1 and 2 (MFN1/2) on the outer mitochondrial membrane and Dynamin-like 120 kDA protein (OPA1) on the inner mitochondrial membrane regulation fusion events, whereas dynamin-related protein 1 (DRP1), its active, phosphorylated form, p-DRP1 and FIS1 regulate mitochondrial fission (DuBoff et al., 2013). Mitochondria with mutations in mtDNA can be kept functioning by undergoing fusion with other mitochondria to compensate for the deficits (Nakada et al., 2001), creating heteroplasmy among mitochondria. Fusion is promoted by energy demand and stress, whereas fission serves as quality control and to generate new organelles (Youle and van der Bliek, 2012).

Fission has been shown to transiently induce hyperpolarization in one mitochondrion and hypopolarization in the other. In case of malfunctioning this would result in complete depolarization which would lead to degradation by mitophagy, a specific form of autophagy in that Drp1 is implicated (Twig et al., 2008). In this process loss of membrane potential leads to accumulation of PINK1 on the outer membrane, leading to phosphorylation of MFN2. This was shown to activate Parkin, an E3 ubiquitin-protein ligase, which then is phosphorylated by PINK1 and ubiqutinates mitochondrial proteins for degradation to set off selective mitophagy (Chen and Dorn, 2013; Müller-Rischart et al., 2013). Mitochondrial dysfunction has been implicated in a range of diseases. In neurons oxidative phosphorylation is the main energy source and needed to maintain axonal transport along microtubules and membrane resting potential (DuBoff et al., 2013).

1.3.2 Mitochondria in Disease

Dysfunction of mitochondria has often been linked to increased ROS production (Alfadda and Sallam, 2012). ROS is mainly produced in complex I and III of the ETC and ROS signaling leads to upregulation of genes involved in mitochondrial biogenesis and quality control, over janus kinase (JNK) and PGC1α (Wallace, 2009). Further, an antioxidant response over NFE2L2 upregulates the expression of proteins such as Glutathione peroxidase, Peroxiredoxins and superoxide dismutase. ROS damage can also result in Ca^{2+}dysregulation, accordingly Ca^{2+} signaling over calcineurin, CAMK, PKC, and JNK leads to expression of calcium metabolism and glycolytic genes. If mitochondria sustain too much damage they undergo mitophagy. This process is regulated by SIRT3, which deacetylates the transcription factor FOXO3a and in turn upregulates mitophagy-associated genes (Papa and Germain, 2014).

Higher levels of ROS have been observed in postmortem brains of AD patients (Mecocci et al., 1994). Aβ and APP were found to be targeted to the mitochondrial complex IV. Aβ-binding alcohol dehydrogenase (ABAD) was shown as a direct target of Aβ that prevented NAD binding and thereby exaggerated oxidative stress (Lustbader et al., 2004). Higher ROS levels were also found in iPSC-derived neurons from familial AD patients, having a E693del mutation and in a sporadic patient. Both were shown to have intracellular Aβ (Kondo et al., 2013). Further, higher levels of cytochrome c oxidase were observed in AD brains, an essential protein in complex IV of the ETC (Hirai et al., 2001; Nagy et al., 1999). Increased levels of mitochondrial fission protein FIS1 and lower levels of DRP1 have been reported, together with decreased levels of OPA1 and MFN1/2 (Wang et al., 2009), leading to impaired mitochondrial dynamics by altered fission and fusion. In addition, abnormal mitochondrial morphology was observed in fibroblasts from AD patients (Wang et al., 2009). Complex I, III, IV and V mRNA levels were higher in the cortex of transgenic mice (Reddy et al., 2004) with higher oxidative stress levels prior to plaque deposition (Yao et al., 2009). Contradicting reports found impaired complex I activity (Derungs et al., 2016; Rhein et al., 2009; Trushina et al., 2012) and

reduced complex III and IV activity (Caspersen, 2005; Rhein et al., 2009).

Mitochondrial dysfunction was shown to be involved in Parkinson's disease (PD), with early onset mutations in PINK1 and Parkin. As described above, malfunctioning of these proteins would lead to deficient mitophagy (Pickrell and Youle, 2015). OPA1 mutations were shown to be responsible for about 50–65% of dominant optic atrophy cases (Yu-Wai-Man et al., 2010). Mutations mostly led to premature mRNA termination and ultimately to loss of the retinal ganglion cell layer in the inner retina (Marchbank et al., 2002; Yu-Wai-Man et al., 2011). In an autosomal dominant form of Charcot-Marie-Tooth disease mutations in MFN2 were identified and shown to lead to progressive sensory loss and muscle weakness (Sottile et al., 2011; Züchner et al., 2004). Mitochondrial dysfunction has a direct causative role in the mentioned diseases, and a role in AD has been well established, yet further evaluation is necessary, especially at an early time point of the disease, at endogenous levels of AD relevant proteins.

1.4 Synaptic plasticity

1.4.1 Synaptic plasticity

The ability of synapses to strengthen or weaken over time is described as synaptic plasticity. This occurs in response to increased or decreased synaptic activity and is essential to learning and memory. Increased activity leads to long-term potentiation (LTP), whereas low levels of activity induce long-term depression (LTD) of synapses. Integral to the process of plasticity are the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) and N-Methyl-D-aspartate receptor (NMDAR). AMPARs are ionotropic transmembrane receptors activated by glutamate. They are tetramers consisting of GluR1, GluR2, GluR3 and GluR4. Permeability to Na^+ or Ca^{2+} depends on the AMPAR subunit composition with GluR2 subunits being present in the majority of AMAPRs and thereby rendering the receptor impermeable to Ca^{2+} (Ashby and McBain, 2007). In LTP, binding of glutamate leads to opening of the central pore and Na^+/Ca^{2+} influx. NMDARs are activated by glutamate binding but additionally require glycine binding to the GluN1 subunit and sufficient depolarization of the neuron for the Mg^{2+}-block of the central pore to be released (Dore et al., 2016). This allows further Ca^{2+} influx. Downstream effects of elevated intracellular Ca^{2+} levels are activation of protein kinase A (PKA), protein kinase C (PKC), and Ca^{2+}/Calmodulin-dependent kinase II (CamKII). Activation of MAPK leads to exocytosis of an intercellular pool of AMPARs and cAMP response element-binding protein (CREB) mediated transcriptional activation of immediate early genes such as cFos (Korte and Schmitz, 2016). Ultimately this process strengthens the synapse and its response to glutamate release from the presynapse. The opposing process, LTD, leads to weakening of synapses and is thought to be critical for fine tuning of established connections. Hereby low levels of Ca^{2+}-influx activate calcineurin and protein phosphatase 1 (PP1) which leads to dephosphorylation AMPARs and their subsequent

internalization by endocytosis (Beattie et al., 2000).

1.4.2 N-methyl-D-aspartate receptors

NMDARs are ionotropic glutamate receptors that are comprised of four subunits. So far, three different subfamilies with in total 7 subunits were identified, GluN1, GluN2A, GluN2B, GluN2C, GluN2D, GluN3A, GluN3B. NMDARs exist as heterodimers with two GluN1 and two GluN2 subunits or heterotrimers with two GluN1 and two different GluN2, or GluN2 and GluN3 subunits. These subunits form a complex with a central pore that is blocked by Mg^{2+} and exhibit an intercellular C-terminal domain (CTD). Release of the Mg^{2+} block requires depolarization of the membrane and glutamate binding (Paoletti et al., 2013). GluN1 has eight isoforms (GluN1-1a – GluN1-4a and GluN1-1b – GluN1-4b) and is ubiquitously expressed throughout the central nervous system (CNS), with GluN1b isoforms mostly restricted to the CA3 region of the hippocampus (Laurie and Seeburg, 1994; Monyer et al., 1994; Vance et al., 2012). The inclusion of different GluN2 subunits has functional consequences for the receptor. Expression of the different subunits changes over the course of development. In embryos GluN2B and 2D are expressed. GluN2A expression starts after birth, together with a decrease in GluN2D. GluN2B expression is maintained in the forebrain. In cerebellum and olfactory bulb, GluN2C expression starts around postnatal day 10 (Akazawa et al., 1994; Sheng et al., 1994). Subunit switch, from NMDARs containing predominantly GluN2B to GluN2A, is vital for synaptogenesis and maturation. This process is thought to be driven by synaptic activity (Barria and Malinow, 2002). In the adult brain, GluN2A is the most prevalent subunit, followed by 2B. Expression of subunits differs between brain regions and neuronal cell types, with further diversity from region specific input (Fritschy et al., 1998; Monyer et al., 1994). Subunit composition reflects differential abilities for conductance, Ca^{2+} permeability, channel opening time, sensitivity to glutamate, glutamate deactivation time and sensitivity to Mg^{2+}. GluN2A subunits were shown to have lower glutamate sensitivity, shorter deactivation time, and a higher maximum probability of finding the channel open, compared to GluN2B (Paoletti et al., 2013). Further examination of the CTDs revealed that GluN2B subunits localize to postsynaptic site by binding to the membrane-associated guanylate kinase, which was not required for GluN2A localization (Prybylowski et al., 2005), as well as latter binding to PSD95, whereas GluN2B preferentially bind to SAP102(Sans et al., 2000). Moreover research showed a stronger affinity of CamKII to GluN2B than GluN2A, which in turn affects synaptic plasticity by increasing LTP levels (Barria and Malinow, 2005).

NMDARs have also been shown to convey processes independent of ion-flux by a metabotropic function. It was shown that induction of NMDAR-LTD did require glutamate binding but was independent of Ca^{2+} flux (Nabavi et al., 2013). Moreover ion flux was not necessary for Aβ-induced LTD (Kessels et al., 2013; Tamburri et al., 2013) and synaptic loss (Birnbaum et al., 2015). Metabotropic NMDAR function was dependent on p38 map kinase activity (Birnbaum et al., 2015).

Further research in the direction of metabotropic NDMAR signaling will be necessary to elucidate its role in physiology and disease.

1.5 Scope of this thesis

Understanding of AD is still incomplete and requires further research. Central to AD is the decline of cognitive function which correlates to the loss of dendritic spines in AD brains. Understanding mechanisms that drive spine loss are essential and NMDARs are thought to be a target of Aβ which may lead to subsequent LTD. NMDARs possess an ionotropic and a metabotropic function. Activation of prior leads to Ca^{2+} influx and thereby activates downstream signaling, whereas latter is independent of ion-flux. Whether spine loss is dependent on Ca^{2+} flux, or depends on metabotropic NMDAR signaling remains unclear. Further, mitochondira are affected in AD and this leads to dysregulation of energy metabolism. This effect can be due to Aβ-mediated stress but to a certain extend also occurs during normal ageing. Due to the inability to research AD in human neurons at an early time point of disease it is not clear whether mitochondrial aberrations are a consequence of disease onset, or also play a causal role.

Therefore the aim of the first part of this thesis is to elucidate the mechanism of Aβ-mediated spine loss. Spefically, it is neccesarry to determine whether metabotropic or inonotropic NMDAR function is active in Aβ-mediated synaptic loss.

In the second part of this thesis, we hypothesize that iPSC-derived neuronal cells from AD patients display aberrant function at an early stage of disease and that altered mitochondrial function is one of the first events in AD. Therefore, skin fibroblasts are reprogrammed to stem cell state and differentiated to neuronal cells. The model of induced neurons is used to determine changes in ROS production, respiratory chain complexs and their correlation to the AD hallmark protein Aβ and tau, as well synaptic changes.

Chapter II

Calcium flux-independent NMDA receptor activity is required for Aβ oligomer-induced synaptic loss

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Author contributions: C.T. and R.M.N. initiated and coordinated the project. J.H.B executed AβO experiments in wt hippocampal slices and C.T. in arcAPP transgenic slices. J.H.B. and C.T. analyzed data. J.B. measured Aβ40. L.R.conributed material for Aβ measurement. C.T. and J.H.B. prepared figures, C.T., and J.H.B. wrote the manuscript.

2.1 Abstract

Synaptic loss is one of the major features of Alzheimer's disease (AD) and correlates with the degree of dementia. N-methyl-D-aspartate receptors (NMDARs) have been shown to mediate downstream effects of the beta-amyloid peptide (Aβ) in AD models. NMDARs can trigger intracellular cascades via Ca^{2+} entry, however also Ca^{2+}-independent (metabotropic) functions of NMDARs have been described. We aimed to determine whether ionotropic or metabotropic NMDAR signaling is required for the induction of synaptic loss by Aβ. We show that endogenous Aβ as well as exogenously added synthetic Aβ oligomers induced dendritic spine loss and reductions in pre- and postsynaptic protein levels in hippocampal slice cultures. Synaptic alterations were mitigated by blocking glutamate-binding to NMDARs using NMDAR-antagonist APV, but not by preventing ion-flux with Ca^{2+}-chelator BAPTA or open channel blockers MK-801 or memantine. Aβ increased the activity of p38 MAPK, a kinase involved in long-term-depression and inhibition of p38 MAPK abolished the loss of dendritic spines. Aβ-induced increase of p38 MAPK activity was prevented by APV but not by BAPTA, MK-801 or memantine treatment highlighting the role of glutamate binding to NMDARs but not Ca^{2+}-flux for synaptic degeneration by Aβ. We further show that treatment with the G protein inhibitor pertussis toxin (PTX) did not prevent dendritic spine loss in the presence of Aβ oligomers. Our data suggest that Aβ induces the activation of p38 MAPK and subsequent synaptic loss through Ca^{2+}-flux- and G protein-independent mechanisms.

Abbreviations: AD: Alzheimer's disease; Aβ: β-amyloid peptide; NMDARs: N-methyl-D-aspartate receptors; LTD: long-term-depression; Mem: memantine; MK: MK-801; PTX: Pertussis toxin; tg: transgenic; p-ERK: phospho-ERK; p-p38: phospho-p38 MAPK; syn: synaptic activation

2.2 Introduction

AD is clinically characterized by cognitive impairments caused by massive neuronal degeneration and synaptic loss. The reduction in synapse numbers is the best neuropathological correlate to the degree of dementia in AD [1]. Besides synaptic alterations the levels of soluble oligomeric forms of Aβ but not plaques correlates best with memory loss in AD [2]. Accumulating evidence indicates that either transgenically produced Aβ or the treatment with Aβ oligomers decrease dendritic spine density [3-6], impair LTP [7], facilitate LTD [8] and induce aberrant spine morphology [5,9].

While the signaling cascades coupling Aβ to synaptic degeneration are incompletely understood, experimental evidence suggests an essential role for NMDARs. Oligomeric Aβ can bind to dendritic spines and treatment with NMDAR antibodies abolishes Aβ binding [10]. Pharmacological inhibition of NMDAR activity also mitigates the pathological effect of Aβ on synapses [4-6,11]. NMDARs are ionotropic receptors permeable for cations and controlled by a voltage-dependent Mg^{2+}-block that is removed after membrane depolarization by α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

receptor (AMPARs). Upon glutamate binding to the NR2 subunit of NMDARs, cations including Ca^{2+} enter the cell. It has been thought for a long time that the levels of Ca^{2+}-influx through NMDARs determine the induction of either long-term-potentiation (LTP, high Ca^{2+}-influx) or long-term-depression (LTD, mild Ca^{2+}-influx) [12]. Nevertheless, a recent study showed that Ca^{2+}-flux is not essential for the induction of NMDAR-LTD whereas glutamate-binding to the receptor is required [13]. NMDAR signaling independently of ion flux has already been proposed to regulate NMDAR phosphorylation and endocytosis [14,15]. Further, the subunit switch between NR2B and NR2A NMDARs is driven by glutamate in the absence of NMDAR currents [16]. However, the role of ion-flux for synaptic loss in AD still remains to be elucidated.

We show that the Aβ-induced pre- and postsynaptic loss is mediated by glutamate binding to NMDARs, independent of ion-influx.

2.3 Results

To determine the mechanisms of synaptic loss by Aβ we cultured hippocampal slices from arcAβ-transgenic mice and infected them with neurotropic Sindbis virus expressing EGFP to visualize single neurons. Neurons in transgenic slices showed reduced dendritic spine densities. Treatment with the NMDAR antagonist D-APV, which blocks the glutamate binding sites, completely abolished spine loss (Fig. 1A, B). This is in agreement with previous findings that the glutamate binding site antagonist CPP rescued spine loss in APP transgenic cultures [5]. Since NMDAR signaling has been reported to be mediated through Ca^{2+}-influx, we sought to determine the role of Ca^{2+}-influx for Aβ-induced loss of dendritic spines. To this end, slices were treated with NMDAR open channel blockers memantine or MK-801 (Fig. 1C) or with Ca^{2+}-chelator BAPTA (Fig. 1E). Neither memantine nor MK-801 nor BAPTA treatment restored spine density in transgenic cultures (Fig. 1D, F). Ca^{2+}-flux through NMDARs requires the removal of the Mg^{2+}-block within the receptor pore, which is achieved upon membrane depolarization by AMPARs. To analyze the involvement of AMPARs we treated cultures with AMPAR antagonist CNQX, but did not observe any effect on spine numbers in transgenic cultures (Fig. 1G, H). Concentrations of inhibitors were chosen according to previous reports demonstrating highest degree of specificity and/or therapeutic relevance (APV and MK-801: [17]; memantine: [18,19]).

To ensure that memantine, MK-801 and BAPTA preparations at the used concentrations are functional and can block NMDARs and the entry of Ca^{2+}, despite having no protective effect, we performed synaptic activation experiments in the presence of these compounds. Synaptic activation induced phosphorylation of extracellular-signal regulated kinases (p-ERK) which is in agreement with previous studies [6,20] (Fig. 1I). The presence of either APV, memantine, MK-801 or BAPTA fully prevented ERK activation, confirming the functionality of these compounds (Fig. 1J). Of note,

memantine blocked synaptic activation although it has been described to preferentially block extrasynaptic over synaptic activity at the used concentration [21].

As shown previously, spine loss in arcAβ-transgenic cultures can be prevented in the presence of anti-Aβ antibodies [6], confirming that Aβ but not APP or any other cleavage product is responsible for the observed effects on spines. Hence, our data indicate that glutamate-binding to NMDARs rather than Ca^{2+}-flux mediates Aβ-induced dendritic spine loss.

To confirm the morphological spine data, we determine whether Aβ also affects the levels of pre- and postsynaptic proteins and analyzed PSD-95 (postsynaptic) and synaptophysin (presynaptic) levels in lysates of non-transgenic and arcAβ-transgenic cultures (Fig. 2A). Compared to controls the levels of both proteins were strongly reduced in transgenic cultures. Treatment with APV but not with memantine or MK-801 rescued the reduction of protein levels (Fig. 2B). Likewise, treatment with BAPTA did not affect loss of synaptic proteins in transgenic cultures (Fig. 2C, D).

Synaptic activity has been shown to increase the production of Aβ and inversely, preventing synaptic activity can reduce Aβ production [22]. To exclude that the protective effect of APV is simply based on Aβ reduction, we quantitatively measured the levels of Aβ40 in the supernatant of arcAβ-transgenic cultures treated with the respective NMDAR antagonists (Fig. 2E) under identical conditions as in the previous experiments. NMDAR antagonists did not significantly alter Aβ levels in the medium of transgenic cultures.

This indicates that, in addition to dendritic spine loss, reductions in pre- and postsynaptic protein levels are caused by NMDAR functions, independent of Ca^{2+}-flux.

A recent study showed that activation of p38 MAPK is essential for Ca^{2+}-independent metabotropic function of NMDARs [13]. We analyzed if p38 MAPK is also involved in the Aβ effects on synapses and examined the activity of p38 MAPK in lysates from non-transgenic and arcAβ-transgenic cultures (Fig. 3A). Increased levels of phosphorylated (active) p38 MAPK (p-p38) were observed in transgenic cultures. After treatment with APV, the levels of p-p38 were reduced to control levels whereas memantine or MK-801 treatment had no effect (Fig. 3B). To ascertain, that active p38 MAPK mediates synaptic deficits caused by Aβ, we treated cultures with the p38 MAPK inhibitor SB239063. Treatment abolished spine loss in arcAβ transgenic cultures (Fig. 3C, D). To examine whether a general increase in synaptic activity in transgenic cultures contributes to increased p-p38 MAPK levels, we treated non-transgenic and arcAβ-transgenic cultures with bicuculline and 4-aminopyridine (Fig. 3E, F). Synaptic activation increased levels of p-ERK. No difference in p-ERK levels were observed between transgenic and non-transgenic cultures, indicating that arcAβ transgenic slices display no general increase in synaptic activity. Further, synaptic activation does not affect the activity of p38 MAPK.

This data suggests that Aβ induces the activity of p38 MAPK, which mediates the loss of dendritic spines. This effect does not depend on Ca^{2+}-influx or general synaptic activation.

Oligomeric Aβ is considered to be one of the main toxic Aβ species in the AD brain. So far, we used cultures from arcAβ transgenic mice to determine the effects of Aβ on synapses in the presence of other APP processing products (Figs. 1-3). Although, arcAβ mice show early formation oligomeric Aβ *in vivo* [23], the role of oligomeric Aβ for our findings requires further investigations. To conclusively validate the role of Aβ oligomers, we treated non-transgenic cultures with defined preparation of Aβ42 oligomers at sublethal concentrations (Fig. 4). Oligomer preparations contained mostly mono-, tri-, and tetramers as determined by silver stained SDS gel and western blot (Fig. 4K) which is in agreement with previous studies [24,25]. Scrambled Aβ, subjected to the same oligomerization protocol as Aβ42, did not aggregate, as expected. Treatment with Aβ oligomers but not scrambled Aβ reduced dendritic spine density to a similar extent as observed in transgenic cultures (compare Fig. 4 and Fig. 1). Confirming the transgenic data, only APV treatment (Fig. 4A, B) but not memantine (Fig. 4C, D), MK-801 (Fig. 4E, F) or BAPTA (Fig. 4G, H) prevented oligomer-induced spine loss. Oligomeric Aβ further reduced PSD-95 and synaptophysin levels, which could not be rescued by BAPTA treatment (Fig. 4I, J). Aβ did not cause cell death at the used concentration (Fig. 4L).

This indicates that oligomeric Aβ, similar to transgenically produced Aβ, exerts its toxic properties on synapses via NMDAR signaling, independent of Ca^{2+}-influx.

To determine whether Ca^{2+}-flux-independent synaptic loss depends on G protein signaling, we treated slices with oligomeric Aβ and pertussis toxin (PTX), an inhibitor of the heterotrimeric $G_{i/o}$ protein family, at concentrations described before in slice cultures [26]. PTX administration did not prevent spine loss caused by Aβ (Fig. 5 A, B) suggesting that Aβ-induced synaptic loss does not require a PTX-sensitive subgroup of G proteins.

2.4 Discussion

In this study, we have examined the role of Ca^{2+}-flux for Aβ-induced loss of dendritic spines and pre- and postsynaptic proteins. Our data show that NMDAR dependent ion-flux is not required for synaptic loss whereas binding of glutamate to the NMDAR is essential for coupling Aβ to synaptic degeneration.

NMDARs have been thought to signal exclusively ionotropic, regulating intracellular signaling via Ca^{2+}-transmission. However, recent evidence indicates that NMDARs can signal metabotropically i.e. independent of ion-flux. The group of Roberto Malinow showed that induction of NMDAR-LTD via activation of p38 MAPK is based on metabotropic signaling [13]. Further, the induction of LTD by Aβ can occur in the absence of Ca^{2+}-transmission [17,27]. Together, these data suggest that glutamate binding

to NMDARs may induce a conformational change that subsequently activates intracellular signaling cascades even in the absence of Ca^{2+}-flux. This possibly does not exclude an additional role of intracellular Ca^{2+}, because the injection of Ca^{2+}-chelators into neurons prevents LTD induction [13,28]. In agreement, oligomeric Aβ has been shown to increase intracellular Ca^{2+} levels by mobilizing Ca^{2+} from the ER rather than promoting influx of Ca^{2+} from the extracellular space [29].

Our data show that the induction of specific metabotropic-like NMDAR signaling pathway by Aβ, which is not induced by general synaptic activation, causes downstream phosphorylation of p38 MAPK. Active p38 MAPK is key player in NMDAR- and mGluR-dependent LTD [30,31] and mediates AMPAR endocytosis [32]. A study by Yang et al described an intracellular pathway based on the co-activation of mGluR5 and NMDARs [33], also independent of Ca^{2+}-flux. Further, mGLuR5 has been implicated in mediating toxic effects of Aβ at synapses [34,35]. Thus, a co-activation of mGluR5 and NMDARs may cause downstream activation of p38 MAPK followed by synaptic loss. However, treatment with the G protein inhibitor PTX did not prevent spine loss in our model, which renders the involvement of mGluRs unlikely.

In previous studies, we showed that caspase-3 and calcineurin are essential for the loss of spines by Aβ [5,6]. Caspase-3 can be activated by p38 MAPK [36,37]. Further, D'Amelio and colleagues reported that caspase-3 and calcineurin mediated synaptic dysfunction in APP transgenic mice. Importantly, they observed that caspase-3 activated calcineurin by proteolytic cleavage in a Ca^{2+}-independent manner, supporting our finding that Aβ-induced synaptic dysfunction can occur in the absence of Ca^{2+}-flux.

An important finding in our study is the lack of synaptic protection by memantine since memantine is the only clinical approved NMDAR antagonist for treatment of AD patients. Memantine, at clinically relevant low µM concentrations, is a low-affinity, uncompetitive open-channel blocker with a relatively high off-rate [38]. High-affinity NMDAR antagonists may be toxic after long exposure due to block of synaptic transmission. However, memantine has been suggested to be more tolerable because of blocking mainly over-excitation of the receptor rather than its physiological activity. As the high off-rate of memantine could still allow Ca^{2+}-influx into the cell, we confirmed the data using a second open-channel blocker MK-801 and the Ca^{2+}-chelator BAPTA. Further, all used compounds could fully block ERK phosphorylation after synaptic activation. Interestingly, treatment of slices with even the high-affinity inhibitors D-APV or MK-801 or with BAPTA did not show any side effects on spines.

Despite having no protective effect for Aβ-induced synaptic loss in our study, memantine may be more beneficial with respect to other Aβ effects. A recent study showed that injection of low-molecular-weight (LMW) oligomers into mice caused persistent memory impairment and synaptic loss whereas injection of high-molecular-weight (HMW) oligomers resulted in neuronal oxidative stress and reversible cognitive deficits but no synaptic loss. Memantine treatment could rescue only the effects of HMW but not LMW Aβ oligomers [39], further indicating that memantine may not be

beneficial with respect to Aβ-induced synaptic alterations. However, memantine protected against the induction of oxidative stress by oligomeric Aβ [10] and studies from our lab showed that memantine, at the concentration used in this study, prevented the increase in tau phosphorylation by Aβ (unpublished observations).

Because synaptic loss occurs early in the disease process [40], our data my contribute to explain why memantine is ineffective in treating early-staged mild AD patients [41]. Together, our data establish a Ca^{2+}-flux- and G protein-independent NMDAR signaling pathway coupling Aβ toxicity to p38 MAPK activation and synapse loss, suggesting pharmacological inhibition of this pathway as a potent mechanism to prevent Aβ-mediated early synaptic loss.

2.5 Materials and Methods

Chemicals / reagents: Cell culture reagents were purchased from Sigma (Schnelldorf, Germany) and Invitrogen (Basel, Switzerland). NMDA receptor antagonists D-APV (also called D-AP5, D-2-amino-5-phosphonovalerate; Batch No.:71), MK-801 ((5S,10R)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate; Batch No.:8), memantine (3,5-Dimethyl-tricyclo[3.3.1.13,7]decan-1-amine hydrochloride; Batch No.:9), Ca^{2+}-chelator BAPTA (1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; Batch No.:4), AMPA receptor antagonist CNQX (6-Cyano-7-nitroquinoxaline-2,3-dione; Batch No.:33) were purchased from Tocris (Bristol, UK). Pertussis toxin was purchased from List Biological Laboratories (Campbell, CA, USA).

Hippocampal slice cultures: ArcAβ-transgenic mice were obtained as described [23]. All animal experiments were performed in accordance with the guidelines of the Swiss veterinary cantonal office. Hippocampal slice cultures were prepared and cultured as described [42]. In short, 6- to 7-d-old transgenic and nontransgenic C57BL/6 mice were decapitated, brains were removed, hippocampi were isolated and cut into 400 µm thick slices. Slices were cultured in culture medium (Minimum essential medium Eagle with HEPES modification, 25% Basal medium with Earle's modification, 25% heat-inactivated horse serum, 2 mM glutamine, 50 units/mL penicillin, 50 µg/mL streptomycin, 0.6% glucose, pH 7.2). Culture medium was exchanged every second or third day. On DIV 11 culture medium was replaced by low-serum Nb-N2 medium (Neurobasal medium, 0.5% heat-inactivated horse serum, 2 mM glutamine, 50 units/mL penicillin, 50 µg/mL streptomycin, 0.6% glucose, 1x N2 supplement, pH 7.2) to ensure more defined condition during analysis. For spine analysis slice cultures were infected with Sindbis virus expressing EGFP on DIV 12 in culture and fixed on DIV15 with 4% paraformaldehyde/sucrose. For protein analysis, uninfected slices were lysed on DIV 15 in culture.

Treatments: To determine inhibitor effects in transgenic cultures, slices were treated with respective inhibitors from DIV 11-15. To analyze effects of oligomeric Aβ, slices were treated with Aβ oligomers or scrambled Aβ from DIV 11-15. To assess the effects of inhibitors on cultures exposed to

oligomeric Aβ, slices were treated with Aβ oligomers and the respective inhibitor in parallel from DIV 11-15. For treatment with pertussis toxin, slices were exposed to PTX from DIV 13-15.

Dendritic spine analysis: To determine dendritic spine density, virus solution was diluted to achieve 1–10 infected neurons per slice to allow imaging of single dendritic fragments. Analysis of dendritic spine density was performed using Leica SP2 CLSM equipped with 63x objective (NA: 1.2) and 488-nm Argon laser. Apical dendritic segments in CA1 *stratum radiatum* were imaged with size of 30 x 30 µm (512 x 512 pixel, voxel size: 0.05813 x 0.05813 x 0.25 µm). Image stacks were processed to maximum projections, and dendritic spine density was determined using ImageJ.
Spine imaging and counting were performed blinded (without the researcher knowing the mouse genotype or culture treatment).

Synaptic activation protocol: Stimulation of synaptic activity was adapted from Tackenberg et al., 2013 [6]. Cultures were pretreated with APV, memantine, MK-801 or BAPTA for 12 hour before activation. Then, cultures were exposed to neurobasal medium containing 1 mM 4-AP, 25 mM bicuculline and the respective inhibitor for 20 min. Control cultures were treated with neurobasal medium containing identical DMSO concentrations as above but devoid of 4-AP, bicuculline and inhibitors.

Western blot: Cultured slices were harvested on DIV 15, sonicated in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.5% deoxycholate, and 0.1% SDS, pH 8.0) containing phosphatase inhibitor cocktails 1 and 2 (Sigma) and protease inhibitor cocktail (Roche, Basel, Switzerland) and centrifuged at 5000 g for 10 min at 4° C. The supernatant was stored at - 80°C. Lysates were subjected to SDS-PAGE followed by immunoblotting using primary antibodies against PSD-95 and Synaptophysin (both Millipore, Billerica, MA, USA, 1:1000), phospho p38 MAPK and p38 MAPK (both Cell Signaling, Danvers, MA, USA, 1:1000), 6E10 (Signet, Dedham, MA, USA, 1:500) and GAPDH (Biodesign, Saco, ME, USA, 1:5000). Band intensities were quantified with ImageJ.

Silver staining: Oligomeric Aβ42 preparations were analyzed by SDS-PAGE. The gel was left overnight in fixing solution (40% EtOH, 10% acetic acid), sensitized in 0.017% sodium thiosulfate for 2 min, impregnated in 0.27% silver nitrate solution (including 0.37% formaldehyde) for 30 min and developed in 0.03 M sodium carbonate (supplemented with 0.15% Formaldehyde and 0.02% sodium thiosulfate). The reaction was stopped in 3% glacial acid.

Aβ oligomer preparations: Synthetic Aβ42 peptide was obtained from American peptide. Preparation of Aβ42 oligomers (Abeta-derived diffusible ligands, ADDLs) was carried out as previously described [43]. Cold 1,1,1,3,3,3-hexafluro-2-propanol (HFIP) was added to Aβ42 peptide to a final concentration of 1 mM. HFIP was evaporated overnight, peptides dried for 10 min in a speedvac and stored at -80°C. Peptides were resuspended in DMSO at 5 mM concentrations. Neurobasal

medium without phenol red was added to achieve a peptide concentration of 100 µM and incubated for 24 h at 4°C. Higher aggregates e.g. fibrils were removed by centrifugation at 14.000 g for 10 minutes at 4°C and the supernatant was used for experimental procedures. Aβ42 oligomer preparations were analyzed by silver staining and western blot for each experiment.

Conflict of interest:

The authors declare that they have no conflict of interest.

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2.7 Figures

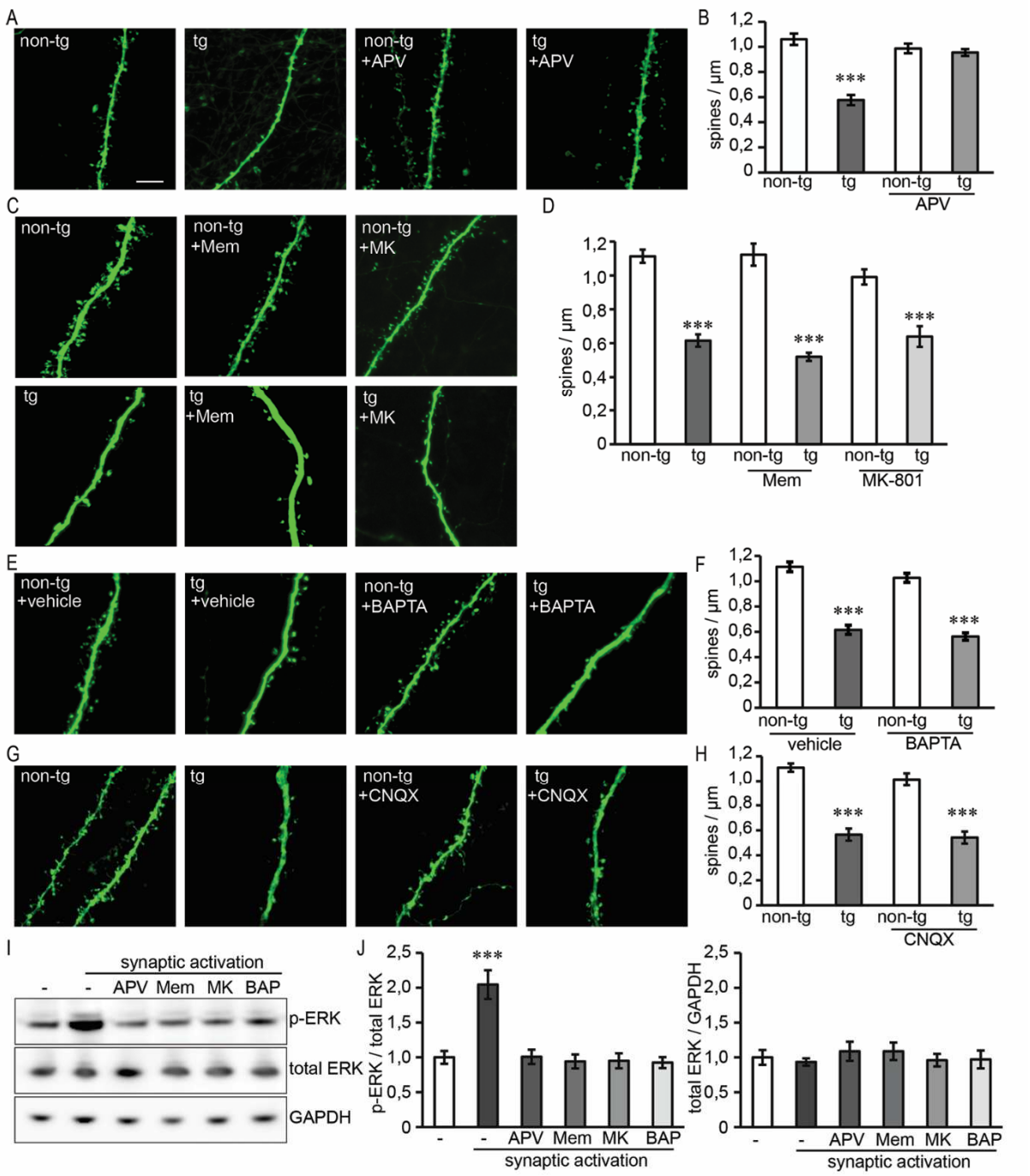

Figure 1 Blocking glutamate binding to NMDARs but not Ca^{2+}-influx prevents dendritic spine loss in arcAβ-transgenic slice cultures.

A: Confocal images of dendrites from CA1 neurons in the stratum radiatum of non-transgenic and arcAβ-transgenic hippocampal slice cultures treated with NMDAR antagonist APV (100 µM). Scale bar: 5 µm **B**: APV treatment reverses the dendritic spine loss in arcAβ-transgenic cultures. n=10-13. **C**: Confocal images of non-transgenic and arcAβ-transgenic cultures treated with NMDAR open channel blocker memantine (1 µM) or MK-801 (30 µM). **D**: Neither memantine nor MK-801 treatment reverses spine loss. n=11-13. **E**: Confocal images of cultures treated with Ca^{2+}-chelator BAPTA (2 mM) or vehicle (BAPTA solvent NaHCO3). **F**: BAPTA treatment does not affect spine loss in transgenic cultures. **G**: Confocal images of cultures treated with AMPAR antagonist CNQX (10 µM). **H**: CNQX treatment does not affect spine loss in transgenic cultures. n=11-15. **I**: Western

blot of lysate from non-transgenic cultures after synaptic activation - in the presence of the reagents used above - showing phosphorylated and total ERK levels. **J**: APV (100 µM), memantine (1 µM), MK-801 (30 µM) and BAPTA (2 mM) pre-treatment prevent ERK phosphorylation after synaptic activation. All values are shown as mean ± SEM; ***p < 0.001; two-tailed unpaired Student's t-test; significances show difference to the respective non-transgenic control (B-H) or to non-activated cultures (J). non-tg: non-transgenic; tg: arcAβ-transgenic; Mem: memantine; BAP, BAPTA; MK, MK-801; p-ERK, phospho-ERK

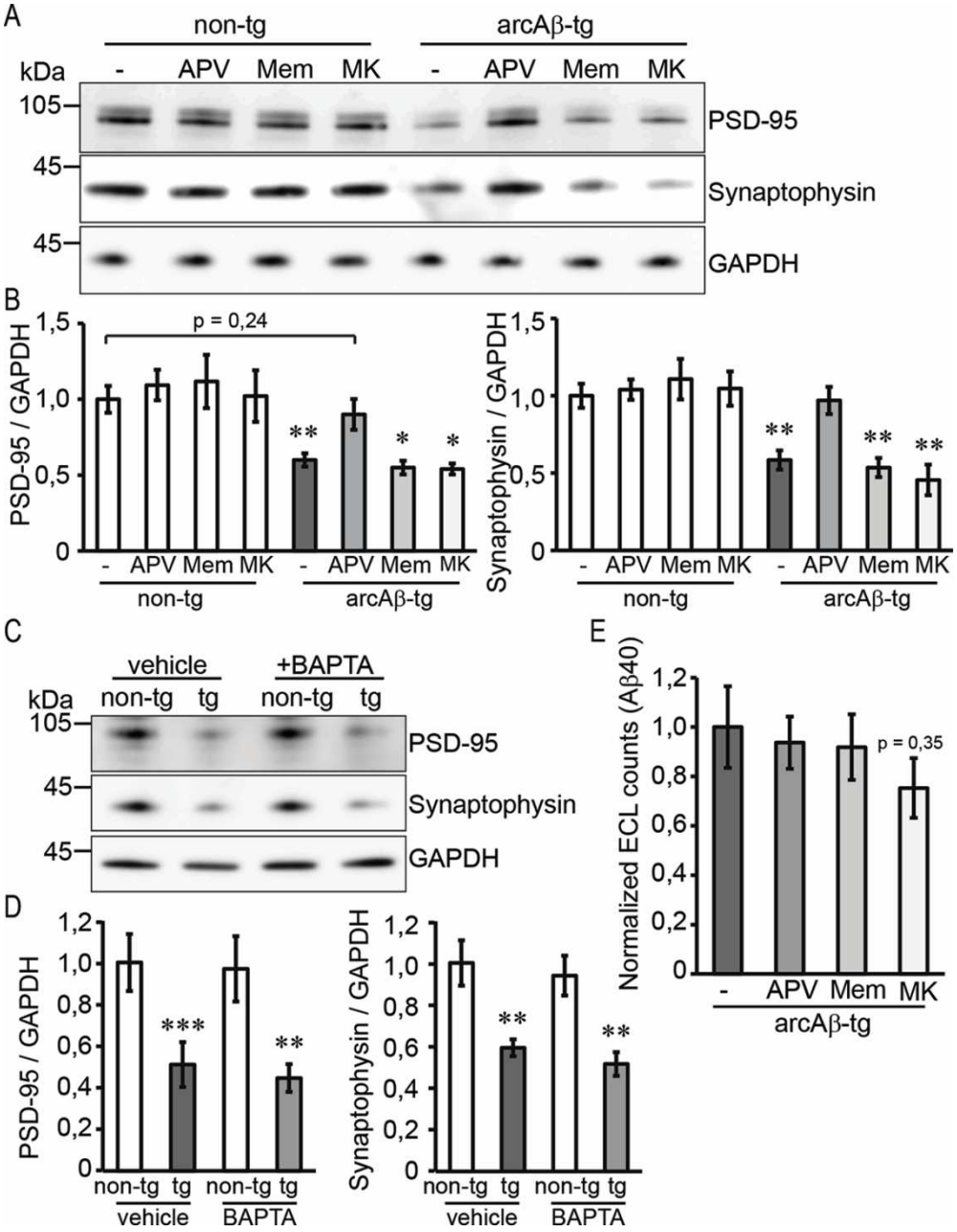

Figure 2 Blocking glutamate binding to NMDARs but not Ca^{2+}-influx prevents the loss of pre- and postsynaptic markers in arcAβ-transgenic cultures.

A: Representative western blot of cell lysates from non-transgenic or arcAβ-transgenic cultures after

treatment with NMDAR antagonists APV (100 µM), memantine (1 µM) or MK-801 (30 µM). **B**: Quantification of western blots. PSD-95 and synaptophysin levels are strongly reduced in arcAβ-transgenic cultures. APV treatment restores PSD-95 and synaptophysin signals back to control levels whereas memantine and MK-801 have no effect. n=6. **C**: Representative western blot of cell lysates from non-transgenic or arcAβ-transgenic slices after treatment with Ca^{2+} chelator BAPTA (2 mM) or vehicle (BAPTA solvent NaHCO3). **D**: BAPTA treatment does not affect loss of synaptic proteins in transgenic cultures. n=6. **E**: Aβ40 levels in the supernatant of arcAβ-transgenic cultures after treatment with NMDAR antagonists measured by MSD. Aβ40 production is not influenced by any NMDAR antagonist. Aβ levels were corrected by protein levels from lysates. n=3.
All values are shown as mean ± SEM (*p < 0.05, **p < 0.01***, p < 0.001; two-tailed unpaired Student's t-test; significances indicate differences to the respective non-transgenic control). non-tg: non-transgenic; tg: arcAβ-transgenic; Mem: memantine; MK: MK-801

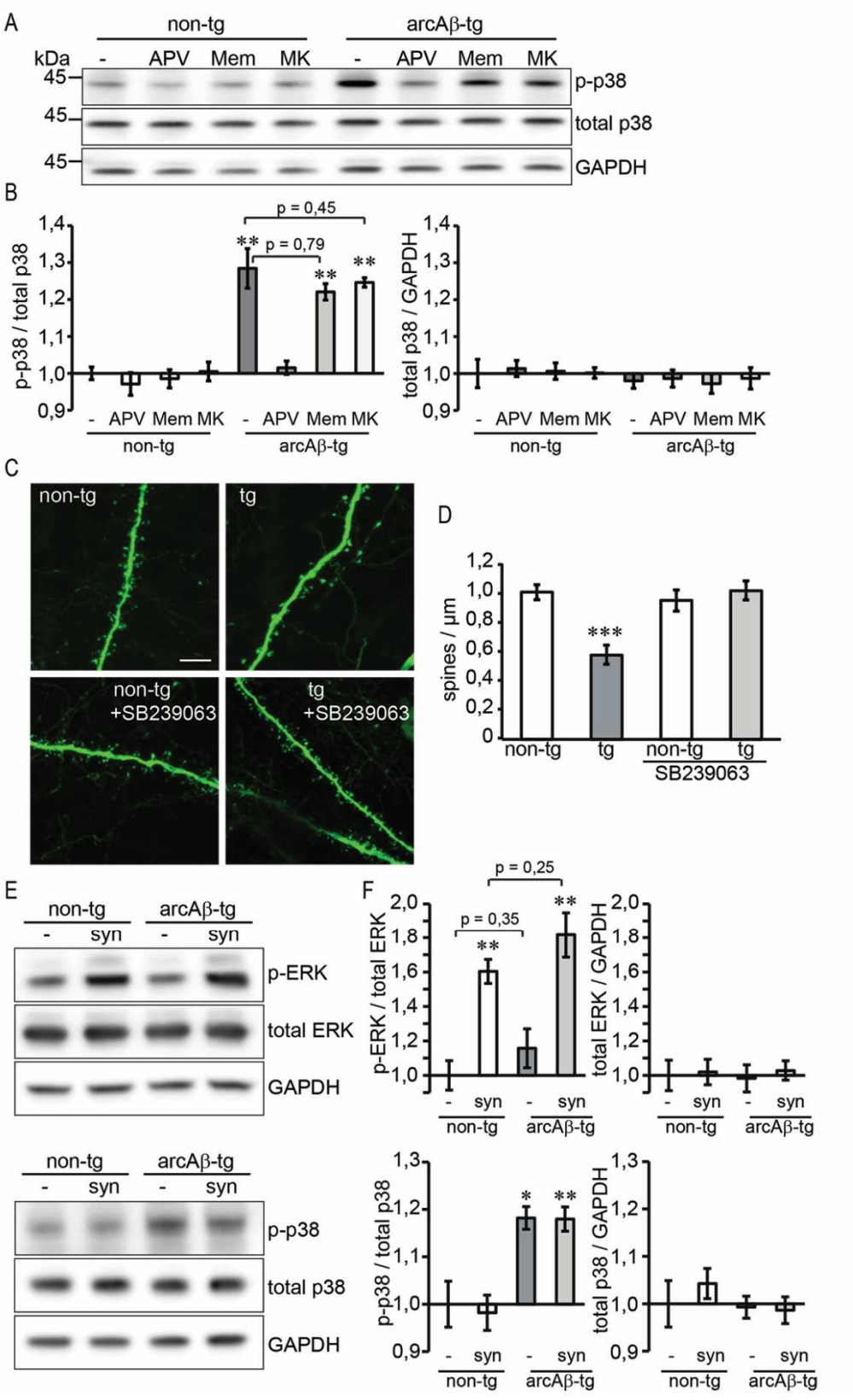

Figure 3 p38 MAPK is activated in arcAβ-transgenic cultures and mediates spine loss.
A: Representative western blot showing phosphorylated (active) and total p38 MAPK in lysates non-transgenic or arcAβ-transgenic slices after treatment with different NMDAR antagonists. **B**: Quantification shows increased levels of phosphorylated p38 in arcAβ-transgenic cultures. The increased amounts of phospho-p38 were reduced to control levels by APV (100 µM) but not by

memantine (1 µM) or MK-801 (30 µM) treatment. The non-transgenic untreated control was set to 1. n=6. **C**: Confocal images of dendrites from CA1 neurons in the *stratum radiatum* of non-transgenic and arcAβ-transgenic hippocampal slice cultures treated with p38 MAP kinase inhibitor SB239063 (20 µM). Scale bar: 5. **D**: SB239063 treatment reverses the dendritic spine loss in arcAβ-transgenic cultures. n=14-16. **E**: Representative western blot showing activated ERK (p-ERK) and activated p38 (p-p38) in non-transgenic or arcAβ-transgenic slices after synaptic activation with bicuculline and 4-aminopyridine. **F**: Quantification shows increased p-ERK levels after synaptic activation, independent of transgenic background. Synaptic activation does not affect p-p38 levels. n=5.
All values are shown as mean ± SEM (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; two-tailed unpaired Student's t-test; significances indicate differences to the respective non-tg control; for p-ERK / total ERK significances indicate differences to the respective non-activated culture). non-tg: non-transgenic; tg: arcAβ-transgenic; Mem: memantine; MK: MK-801; p-ERK: phospho-ERK; p-p38: phospho-p38 MAPK; syn: synaptic activation

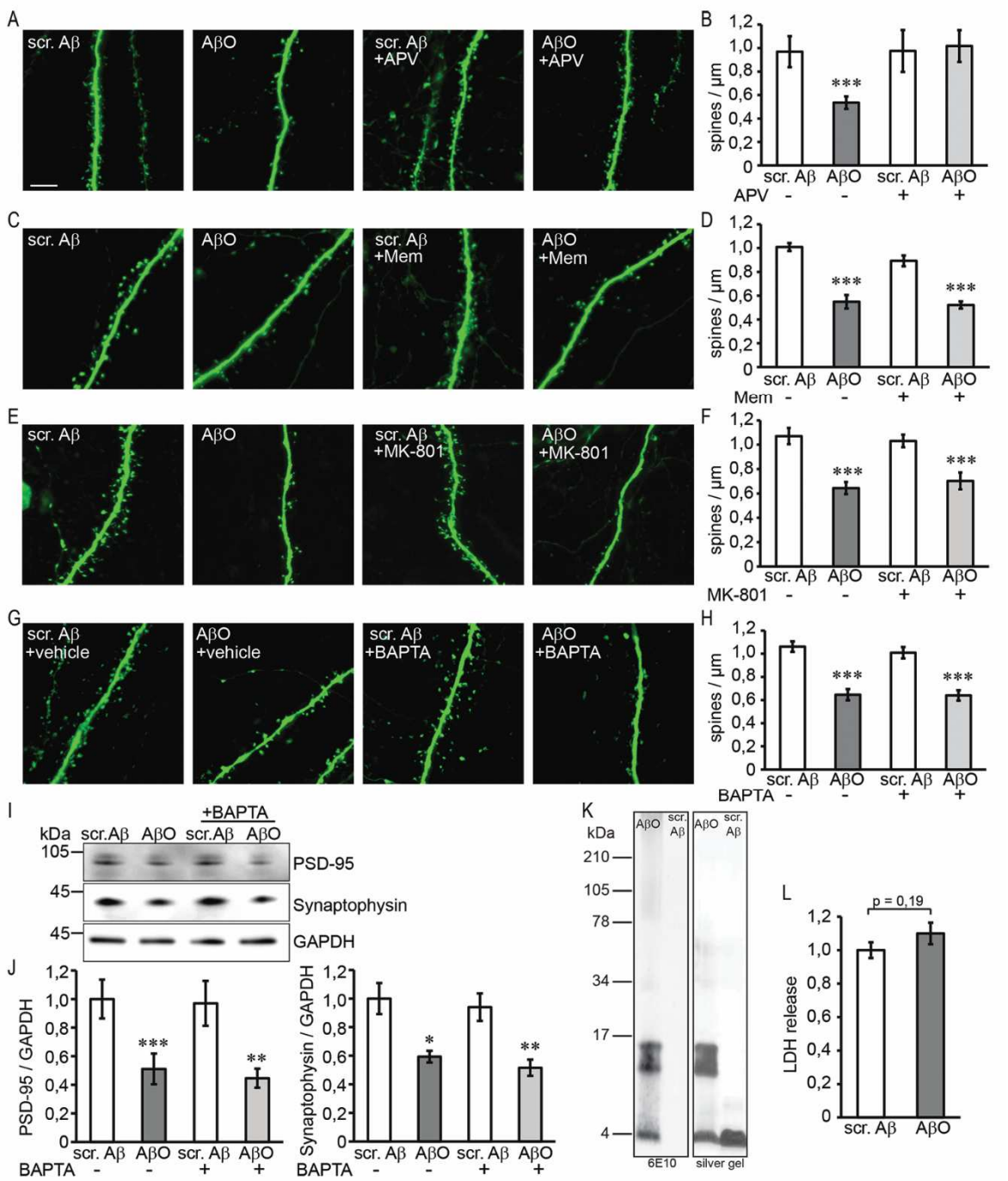

Figure 4 Oligomeric Aβ-induced synaptic loss is prevented by APV but not by memantine, MK-801 or BAPTA treatment.

A: Confocal images of dendrites from CA1 neurons in the *stratum radiatum* of non-transgenic slice cultures treated with oligomeric Aβ (500 nM) or scrambled Aβ (500 nM) and NMDAR antagonist APV (100 µM). Scale bar: 5µm. **B**: APV treatment prevents Aβ oligomer-induced dendritic spine loss. n=13-17. **C**: Confocal images of non-transgenic cultures treated with oligomeric Aβ (500 nM) and NMDAR antagonist memantine (1 µM). **D**: Memantine treatment does not prevent spine loss. n=12-17. **E**: Confocal images of cultures treated with oligomeric Aβ (500 nM) and NMDAR antagonist MK-801 (30 µM). **F**: MK-801 treatment does not prevent spine loss. n=13-15. **G**: Non-transgenic cultures treated with Aβ oligomers (500 nM) and Ca^{2+}-chelator BAPTA (2 mM) or vehicle (BAPTA solvent $NaHCO_3$). **H**: BAPTA does not prevent spine loss caused by oligomeric Aβ. n=11-15 **I**: Representative western blot of cell lysates from slices after treatment with Aβ oligomers (500 nM) and

Ca^{2+}-chelator BAPTA (2 mM). **J**: BAPTA does not prevent reduction in PSD-95 or synaptophysin levels after Aβ oligomer treatment. n=6. **K**: SDS-Gel showing oligomeric Aβ preparations and scrambled Aβ after silver staining (right panel) and western blot stained with 6E10 antibody (left panel). Monomers, tri- and tetramers are observed in the oligomeric preparation, whereas scrambled Aβ only shows monomers. **L**: LDH assay showing no toxicity of Aβ oligomer treatment (500 nM) compared to scrambled Aβ. n=6.
Values are shown as mean ± SEM (*p < 0.05, **p < 0.01***, p < 0.001; two-tailed unpaired Student's t-test). Scr. Aβ: scrambled Aβ; AβO: oligomeric Aβ; Mem: memantine

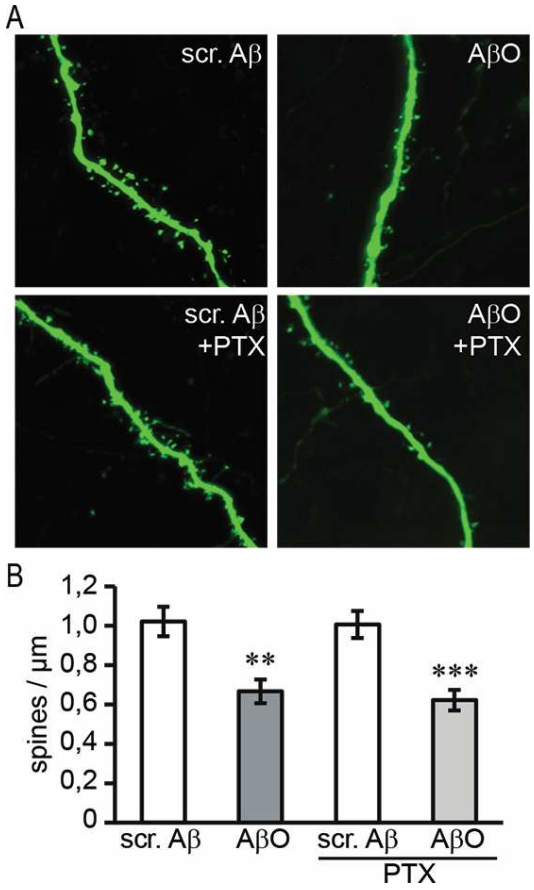

Figure 5 Oligomeric Aβ-induced synaptic loss is not prevented by treatment with PTX.
A: Confocal images of dendrites from CA1 neurons in the *stratum radiatum* of non-transgenic slice cultures treated with oligomeric Aβ (500 nM) or scrambled Aβ (500 nM) and G protein inhibitor PTX (500 ng/ml). **B**: PTX treatment does not prevent Aβ oligomer-induced dendritic spine loss. n=12. Values are shown as mean ± SEM (**p < 0.01***, p < 0.001; two-tailed unpaired Student's t-test). Scr. Aβ: scrambled Aβ; AβO: oligomeric Aβ; PTX: Pertussis toxin

Chapter III

Oxidative stress and altered mitochondrial protein expression in the absence of amyloid-β and tau pathology in iPSC-derived neurons from sporadic Alzheimer's disease patients

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Author contributions: C.T., R.M.N and J.H.B initiated and R.M.N and C.T. coordinated the project. A.F.G., A.S. and C.H. were responsible for ethical approval, patient recruitment and skin biopsies. J.H.B reprogrammed cells, maintained iPSCs and assessed DNA damage. J.H.B, D.W. and C.T. characterized iPS cells. D.W. and J.H.B differentiated cells, did qRT-PCRs and MSD measurements. J.H.B and D.W. did western blots and analyzed data. J.H.B., D.W. and C.T. did CellRox assays. J.H.B prepared figures, J.H.B and C.T wrote the manuscript.

3.1 Abstract

Mitochondrial dysfunction is a prominent feature of Alzheimer's disease (AD) and increased production of reactive oxygen species (ROS) has been described in postmortem brain samples and animal models. Moreover, altered mitochondrial complex composition and imbalances in mitochondrial dynamics, namely fission and fusion, have been reported. However, these observations were made at a late stage of disease and the inability to examine an early, presymptomatic phase in human neuronal cells impeded our understanding of cause and consequence of mitochondrial dysfunction in AD. We used human iPSC-derived neuronal cells (iN cells) from sporadic AD (SAD) patients and healthy control subjects (HCS's) to show aberrant mitochondrial function in patient-derived cells. We observed that neuronal cultures from some patients produced more ROS and displayed higher levels of DNA damage. Furthermore, patient-derived cells showed increased levels of oxidative phosphorylation chain complexes, whereas mitochondrial fission and fusion proteins were not affected in this model of early stage AD. Surprisingly, these effects neither correlated with Aβ nor phosphorylated and total tau levels. Synaptic protein levels were also unaffected in SAD and HCS iN cells. The results of this study give new insights into neuronal changes in a presymptomatic phase of AD and suggest an integral role for mitochondrial dysfunction in AD even before the appearance of amyloid and tau pathology.

3.2 Introduction

Alzheimer's disease is the most prevalent neurodegenerative disease and characterized by the appearance of amyloid-plaques and neurofibrillary tangles in the human brain (Winblad et al., 2016). AD is classified in an early-onset, familial type (FAD) and a late-onset, sporadic type, which represents about 95% of the cases (Winblad et al., 2016). The cause for FAD are mutations in APP, PSEN1 or PSEN2, whereas the underlying mechanisms in SAD are still not clear. Genome-wide association studies (GWAS) have identified around 20 single nucleotide polymorphisms (SNPs) for SAD risk but provided little information about biological processes (Deming et al., 2017; Hopkins et al., 2015; Huang et al., 2017a; Sims et al., 2017). Further, dysregulation of an entire pathway can be caused by mutations dispersed throughout the genome, yet these might not be identified due to their different locations. Induced pluripotent stem cells offer a new venue to AD research, not only due to the ability to model sporadic diseases but also because they allow the use of human cells. Thus far, multiple studies used iPSC-derived neurons to assess AD pathology and possible treatments. Drug testing was applied to elucidate mechanisms of APP processing (Brownjohn et al., 2017; Mertens et al., 2013) and several groups used iPSCs with familial AD mutations to show increased Aβ production and tau phosphorylation (Moore et al., 2015; Woodruff et al., 2013; Yagi et al., 2011) caused by these mutations. Two of the first studies using AD patient-derived neuronal cells were comparing SAD to FAD-derived cells (Israel et al., 2012; Kondo et al., 2013). Israel and colleagues used lines from two

patients with APP duplications and two SAD patients with one SAD line behaving like non-demented controls and the second line similar to FAD lines, in regard to Aβ production, p-tau and active GSK-3-β levels. Kondo *et al.* used two different familial mutations and SAD patient cells. APP-E693Δ and one SAD line showed higher ROS levels and intracellular Aβ.

Mitochondrial dysfunction such as increased oxidative mtDNA damage (Hirai et al., 2001; Mecocci et al., 1994) or higher cytochrome oxidase levels (Hirai et al., 2001; Nagy et al., 1999) have been hypothesized to be the potential causes of oxidative stress in AD brains. Also, an increase of mitochondrial fission protein Fis1 with concurrent downregulation of Drp1 and fusion proteins Mfn1/2 and OPA1 has been reported (Wang et al., 2009). In mouse models of familial AD reduced oxidative and complex I activity has been found (Derungs et al., 2016; Rhein et al., 2009; Trushina et al., 2012), as well as impaired complex III and IV activity (Caspersen, 2005; Rhein et al., 2009). Whereas a contradicting report found higher mRNA levels of complex I, III, IV and V in the cortex of Tg2576 mice (Reddy et al., 2004). Observations in triple transgenic mice showed increased oxidative stress levels prior to plaque deposition (Yao et al., 2009). In conclusion, mitochondrial dynamics and function are affected in AD, yet it remains difficult to delineate a clear mechanism due to a lack of human material to examine an early timepoint of the disease and because findings in mouse models might be affected by overexpression artifacts (Saito et al., 2016; 2014).

Where prior studies focused on Aβ-mediated pathology, we aimed to examine mitochondrial dysfunction at an early stage of disease. In this study we show that iPSC-derived induced neuronal cells from SAD patients show in part increased ROS production and display higher levels of oxidative phosphorylation chain (oxphos) complexes. These effects did not correlate with Aβ and tau levels.

3.3 Results

To address the mechanisms of oxidative stress and mitochondrial dysfunction in SAD neuronal cells, we harnessed the iPSC technology (Takahashi et al., 2007) to reprogram AD patient and HCS fibroblasts to pluripotency and differentiated those to induced neuronal cells (iN cells) (Zhang et al., 2013). AD subjects were diagnosed according to standard diagnostic criteria (McKhann et al., 2011), had a documented decline over time, underwent complete clinical work up and neuropsychological testing and had either positive amyloid-PET (11-C-PiB) signal or a typical CSF constellation (Table 1). They were compared to HCSs from the same age group with a negative amyloid-PET.

Fibroblast samples isolated from skin biopsies were reprogrammed in feeder-free conditions (Chen et al., 2011) by delivery of episomal plasmids (Okita et al., 2011). IPSC clones were analyzed for an embryonic stem cell-like morphology, normal karyotype and expression of pluripotency markers Tra-1-60 and Nanog (Figure 1A and Figure S1). Two clones per individuum, positive for all criteria, were used for subsequent analysis. Neuronal differentiation was carried out by lentiviral delivery of doxycycline-inducible Neurogenin2 (Ngn2) expression (Zhang et al., 2013). Neuronal cells underwent

morphological changes over the course of 21 days (Figure 1B), expressed the neuronal marker Map2, displayed synaptic boutons (Figure 1C) and showed spontaneous activity in calcium imaging (data not shown). Cells were co-cultured with mouse glia cells to support survival and synapse formation (Zhang et al., 2013).

Various reports show widespread changes in mitochondrial function in AD. The production of ROS in mitochondria was found to be augmented in AD human samples and mouse models (Mecocci et al., 1994; Yao et al., 2009) and might play an integral role in disease (Grimm et al., 2015). To analyze whether AD patient-derived neurons display higher levels of oxidative stress than HCS neurons we used confocal imaging of CellRox green, a dye that increases fluorescence upon ROS-mediated oxidation. To avoid confounding with glia-derived ROS we measured CellRox intensities in NeuN positive puncta. Menadione treatment was used as a positive control for ROS production. Analysis showed higher ROS levels in AD2, AD3, and AD4 iN cells showing statistical significance compared to both HCS's (Figure 2A,B). Results of individual clones are shown in Figure S2. These findings suggested altered mitochondrial function in at least three out of five AD patients.

Increased ROS production may be caused by dysfunctions of the mitochondrial respiratory chain. Assessment of protein levels of oxphos chain subunits are an indicator for altered complex function. Using an oxphos antibody cocktail we showed higher NDUFB8 protein levels, a subunit of oxphos complex I, in AD4 patient cells, compared to both control lines (Figure 3A,B). We further observed higher protein levels of UQCRC2, a complex III subunit, in cells from AD4 and AD5 compared to HCS1 (Figure 3A,C). The most striking changes in oxphos chain composition were observed for MTOC1, a complex IV subunit. All AD-derived iN cell lines had higher MTOC1 levels than HCS1, and AD4 and AD5 additionally showed significant higher MTOC1 levels than HCS2 (Figure 3A,D). Complex V subunit ATP5A showed higher protein amounts in AD2 and AD4 compared to HCS1 (Figure 3A,E).

Misbalance in mitochondrial dynamics, i.e. fusion and fission, affects mitochondrial integrity and may result in the observed changes in ROS levels (DuBoff et al., 2013). Thus we assessed whether mitochondrial fission and fusion protein levels were altered in AD patient-derived iN cells. Mfn1, Mfn2 and Opa1 regulate mitochondrial fusion. We used western blotting to assess Mfn1 levels but did not observe an alteration in AD-patient iN cells (Figure 3F,G). Likewise we examined Mfn2 and found no changes in patient cells (Figure 3F,H). Opa1 mRNA levels, a protein mediating mitochondrial inner membrane fusion, were determined by q-RT-PCR. We found decreased mRNA levels in AD2 cells compared to HCS1, whereas other cell lines showed comparable mRNA levels (Figure 3I). Regulators of mitochondrial fission are Drp1, its active from phosphorylated-Drp1, and Fis1. We could not observed differences between HCS-derived and patient iN cells in regard to total Drp1 (Figure 3J,K) and phosphorylated-Drp1 protein levels (Figure 3J,L). Further, q-RT-PCR of *Fis1* showed similar mRNA levels in control and patient cells (Figure 3M). Individual clones are depicted

in Figure S3. Examination of mitochondrial function suggests that AD patient-derived iN cells, which are supposed to mimick early changes in AD, are rather affected by altered oxphos complex composition than by defects in mitochondrial dynamics.

Aggregates of Aβ and tau are the major hallmarks of AD and thought to be the main driver of AD pathology. Evidence exists that both, Aβ and tau, affect mitochondrial function and thereby may contribute to ROS generation (DuBoff et al., 2013; Rhein et al., 2009). Therefore we examined whether AD-patient derived iN cells show differences in APP processing, Aβ secretion, as well as tau and phospho-tau levels. We further investigated whether these correlate with the observed misregulation of mitochondrial proteins or increased ROS production in AD patient iN cells. First we assessed levels of APP protein in iN cells from HCS's and AD-patients. As expected from samples of sporadic AD cases, no higher total APP levels could be observed, in fact AD5 APP levels were lower compared to HCS2 (Figure 4A,B). Amylogenic processing of APP by β-secretase leads to the generation of C99 (β-CTF), whereas α-processing generates C83 (α-CTF). We assessed if ratios and total abundance of these products were changed in AD-derived iN cells. We detected slightly higher C99/C83 ratios in AD1 to HCS2 cells, other cell lines showed no change in processing ratios (Figure 4C). Absolute C99 and C83 levels did not differ between control and AD cells (Figure 4D,E and S4C,D). As further processing of C99 by γ-secretase (de Strooper, 2010) leads to production of different Aβ species, we examined the ratios of Aβ42 to Aβ40 in HCS and AD-patient-derived iN cells. Supernatant of iN cells was conditioned for 7 days and subsequently used for MSD analysis. Aβ42 is regarded as the most toxic species and its ratio to Aβ40 is an essential determinant of amyloid aggregation in AD (McGowan et al., 2005). MSD analysis showed no difference in Aβ42 to 40 ratios, except for cells from patient AD3, which displayed a lower ratio compared to HCS1 (Figure 4F). Analysis of absolute Aβ levels showed higher total Aβ40 and 42 in AD5 iN cells compared to HCS2 and lower levels of Aβ40 and Aβ42 in AD3 compared to HCS1 (Figure S4E,F). Analysis of total and phosphorylated tau by western blot showed no differences between AD and HCS iN cells (Figure 4G-I and S4G,H). Taken together, APP processing and tau modification are not altered in AD patient iN cells. The increase in total Aβ levels in the AD5 cell lines does not correlate with the findings of ROS formation in AD cells, as AD5 iN cells did not show increased ROS levels.

Oxidative stress and dysregulation of mitochondrial proteins can induce downstream effects, such as DNA damage (Quirós et al., 2016) or synaptic dysfunctions (Kamat et al., 2014). Phosphorylation of histone H2AX has been identified as marker of DNA double-strand breaks (Rogakou et al., 1998). Immunocytochemistry of phosphorylated γ-H2AX in neuronal nuclei, stained by NeuN, revealed slight but significantly higher DNA damage in iN cells from patient AD2 and AD4 compared to HCS2 but not to HCS1 (Figure S5A,B).

Loss of synapses is the best correlate to the onset of cognitive decline in AD (Terry et al., 1991). We set out to examine levels of different pre- and post-synaptic proteins in HCS and AD-derived iN cells.

Levels of synapsin1, a marker of the presynapse, was found to be similar in iN cells from patients and controls (Figure S6A-C). Analysis of PSD95, a major scaffold in the postsynapse, showed lower levels in AD1 cells compared to HCS2 (Figure S6D-F). DrebrinA, an actin binding protein involved in recruitment of PSD95 to postsynapses (Takahashi et al., 2003) is downregulated in AD brains (Shim and Lubec, 2002). In line with our previous results, comparison of patient and control lines displayed no significant changes in protein levels (Figure S6G-I). Taken together, no major synaptic alterations between HCS's and AD patients were found, suggesting that these alterations may depend on Aβ and arise later in the disease (Birnbaum et al., 2015; Palop and Mucke, 2010).

3.4 Discussion

In this study we examined disease pathology in iPSC-derived neuronal cells from clinically well-defined AD patients and healthy control subjects. Using a rapid neuronal differentiation protocol we were able to examine pure human neuronal cells, without culture impurities from other human cell types, in contrast to differentiation protocols which were used previously to examine SAD pathology {Israel:2012hi, Kondo:2015vf}. The use of iN cells allows investigation of early AD, before onset of amyloid and tau pathology. We showed that some patient-derived iN cells display increased ROS production and aberrant mitochondrial complex composition, which did not correlate with Aβ, tau or p-tau levels. Our findings suggest a strong involvement of altered mitochondrial function in early AD. Increased ROS levels have been observed in various AD models (Hauptmann et al., 2009; Hyun et al., 2010; Reddy et al., 2004; Rhein et al., 2009) and were found to be implicated in other neurodegenerative processes (Busciglio, 1995; Liu et al., 2015; Zuo and Motherwell, 2013). ROS production often promotes DNA damage and vice versa (Quirós et al., 2016) and we also observed increased DNA damage in our samples. The relation between ROS and DNA damage is supported by the finding that the AD cell lines with highest ROS levels also showed the highest degree of DNA damage. We further analyzed the composition of the mitochondrial oxidative phosphorylation chain, with complex I and III of the respiratory chain being the main sites of ROS production. We found an upregulation of complex IV subunit MTOC1 in all and upregulations of complex I, III, and V in certain AD-derived iN cells. Cells from patient AD4 showed higher levels of complex I and III, which could explain the increased ROS levels. However, in AD2 and AD3 patient-derived iN cells, which also showed increased ROS production, no changes in these complexes were seen. Thus, ROS production here might be caused by an alternative mechanism, with complex IV upregulation as part of an oxidant defense mechanism (Turrens, 2003). The changes in oxphos protein levels were in line with reports showing an upregulation in complex I, III, IV and V in brains of AD patients (Nagy et al., 1999) and Tg2576 mice (Reddy et al., 2004), whereas contradicting reports found lower levels of cytochrome C oxidase (COX) in complex IV (Bosetti et al., 2002; Pérez-Gracia et al., 2008). We argue that our findings of complex IV upregulation may represent compensatory mechanisms, in order to

account for reduced functionality. Mitochondrial fission and fusion abnormalities by assessment of MFN1/2, OPA1, DRP1, phosphorylated-DRP1 and FIS1 levels could not be detected in our samples, with the exception of AD2 cells showing lower OPA1 mRNA levels. Previous studies reported reduced OPA1, MFN1, and MFN2 levels and higher FIS1 levels in human AD brain tissue. Drp1 analysis has been reported to be lower in AD tissue in one study and higher levels in another (Manczak and Reddy, 2012; Wang et al., 2009). As we observed a stronger effect on complex dysregulation than changes in fission and fusion, we speculate that these changes in AD brains may arise later in disease and are not visible at an early stage. Examination of APP processing is in line with prior observations that familial AD patients suffer from increased APP levels (APP duplication) or increased Aβ production, whereas sporadic patients mostly have normal APP and Aβ levels (Israel et al., 2012; Kondo et al., 2013). Yet, the strongest risk factor for sporadic AD ApoE4 has recently been linked with higher APP transcription in human stem cell-derived iN cells (Huang et al., 2017b). We can rule out ApoE derived effects in our system, as mouse glia, that is used for co-cultures in our system, only produces one ApoE isoform. Abnormal tau phosphorylation has been linked with tangle formation and neurodegeneration (Goedert et al., 1989; Tackenberg and Brandt, 2009; Xia et al., 2017; del C. Alonso et al., 1996). We could not detect changes in tau phosphorylation, whereat it is important to note that iPSC-derived iN cells only express 3R and no 4R tau (data not shown), which does not reflect the situation in adult neuronal cells (Sposito et al., 2015). We cannot rule out that the observed effects are due to the different gender of controls and patients. However, heterogeneity between AD patients makes it unlikely that the examined differences are gender effects, as observed in AD1 cells, which perform similar to HCS's. Further, studies of mitochondria of male and female mice showed no difference in bioenergetics, oxidative stress and apoptosis (Sanz et al., 2007). Moreover, inherited mitochondrial mutations were found to affect male ageing, but not female (Camus et al., 2012) and male neurons were more negatively affected by starvation, than female neurons (Du et al., 2008). Further, epigenomic effects might have contributed to AD progression, which cannot be detected in reprogrammed cells (Lunnon et al., 2014; de Jager et al., 2014), or that some of the phenotypes we observed are due to reprogramming induced mutations (Kilpinen et al., 2017).

This study focused on cortical population of neurons, as a first step to uncover cellular changes of this complex brain disorder. Yet, it is known that also other cell types such as astrocytes and microglia play a crucial role in AD, as well as vascular deficits (Derungs et al., 2016; Merlini et al., 2016; Merlini et al., 2011; Paolicelli et al., 2017). Systems based on iPSC-derived cells allow uncovering contributions of different cell types to AD pathogenesis in a controlled fashion. Multiplexed single-cell analysis will help to clarify complex cellular rearrangements in early disease. Our study showing oxidative stress and mitochondrial aberrations in iPSC-derived AD patient neuronal cells, which captures an early phenotype of the disease, raises further questions. A more in depth analysis of mitochondrial changes will be necessary to show if these changes converge at a certain pathway and how this affects energy metabolism, mitochondrial localization and transport, and ultimately neuronal

function. Cellular mitochondrial changes might also exert effects on surrounding tissue, creating a local dysregulated environment. Prior studies using iPSC-derived neurons focused on Aβ- and tau-mediated effects in SAD and FAD patient-derived cells (Israel et al., 2012; Kondo et al., 2013). Here we reported increased ROS generation and mitochondrial complex levels in patient-derived iN cells, which does not correlate with Aβ or tau levels. Thus, these AD-relevant mitochondrial aberrations might have a causative role in disease development in early stages preceeding amyloid and tau pathology or they might render cells more susceptible for Aβ- and tau-mediated neurodegeneration in later stages of the disease.

3.5 Methods

Culture and reprogramming of human fibroblasts

Fibroblasts were obtained from skin biopsies. Samples were reduced to small pieces and plated in hFib medium (DMEM (ThermoFisher Scientific) + 10% FCS + 2 ng/ml bFGF (Peprotech)). Fibroblasts were split by trypsination and transduced with reprogramming plasmids at passage 3 (P3). A total of 1 µg of plasmids pCXLE-hUL, pCXLE-hSK and pCXLE-hOCT3/4-shp53-F (Addgene: 27080, 27078, 27077)(Okita et al., 2011) were delivered by nucleofection with the Neon transfection system (ThermoFisher Scientific) to 10^5 cells using the following settings: 950V, 40ms, 2x. Cells were plated in hFib medium supplemented with 0.1 µM sodium butyrate (Sigma). Medium was changed every other day. On day 7 cells were replated on growth factor reduced matrigel (200µg/ml, Corning) in hFib medium + 0.1 µM sodium butyrate + 2 µM Thiazovivin(Sigma). Next day medium was changed to E7 (Stemcell Technologies)(Chen et al., 2011) + 0.1 µM sodium butyrate and changed every other day until d21. iPSC colonies were picked under a stereomicroscope and expanded in E8 medium (ThermoFisher Scientific)(Chen et al., 2011). Ten colonies of each patient and control subject were picked and characterized (see Figure 1 and S1) and two of these colonies (clones) were used for subsequent experiments to account for clonal variation. Main figures show the data of two clones pooled to represent the changes in a patient or control subject. Data for individual clones are displayed in the supplementary figures.

Maintenance of iPSCs

IPSCs were maintained in E8-flex medium (ThermoFisher Scientific) on growth factor reduced matrigel (Corning). Cells were split once to twice a week with ReLeSR (StemCell Technologies), according to manual.

Differentiation of iPSCs to iN cells

Cells were differentiated according to Zhang et al. (2013), with slight modifications. On day -2 iPSCs were dissociated with Accutase (ThermoScientific) and 9 – $20x10^4$ cells (depending survival of the cell line) plated on matrigel coated coverslips in a 24-well plate in E8 supplemented with Thiazovivin

(2 µM, Sigma). Cells were infected with lentiviruses expressing rtTA and Neurogenin2 on day -1 in fresh E8 medium. On day 0 medium was changed to induction medium (DMEM/F12 (ThermoScientific) with doxycyclin (2 µg/ml), 1x N2 (ThermoFisher), 1x NEAA (Sigma), BDNF (10 ng/ml, Peprotech), NT-3 (10 ng/ml, Peprotech) and Laminin (200 µg/ml, Sigma)). On day 1, induction medium was changed and supplemented with Puromycin (1µg/ml, Sigma) for a 24h selection period. On day 2, 70.000 glia cells were added in iN-Neurobasal medium (Neurobasal-A (ThermoFisher) with 1x B27 (Gibco), 2 mM Glutamax (Gibco), 2 µM AraC (Sigma), 2 µg/ml Doxycyclin, 10 ng/ml BDNF, 10 ng/ml NT-3, and 200 µg/ml Laminin)). 50% of the medium was changed every other day until day 10, when medium was changed to Neuronal medium (MEM (ThermoFisher), 1x B27, 0.5% Glucose, 0.02% $NaHCO_3$, 100 µg/ml Transferrin (Sigma), 5% FCS, 0.5 mM l-glutamine and 2 µM AraC). Neuronal medium was changed twice a week until cells were assayed on day 21 – day 23.

Glia preparation and maintenance

Glia cells were prepared from P1 wild-type CD1 pups as followed. Brain was extracted, meninges removed and forebrain isolated. Forebrains were digested in 10 U/ml papain for 30 min and cells dissociated by pipetting. Cells were plated in DMEM with 10% FCS in t75 flasks. Glia were split 1:3 with trypsin at 80 % confluence. Glia was used for co-cultures between passages 2-4.

Lentiviral production

HEK293T cells were maintained in DMEM with 10% FCS and split every other day by trypsination. Lentiviral vectors including three helper plasmids were delivered by calcium phosphate transfection[(Chen and Okayama, 1987)]. In brief, $5x10^7$ cells per 15 cm dish were plated for transfection. 12.5 µg VSV-G, 25 µg pMDLg/pRRE, 12.5 µg pREV and 50 µg plasmid of interest were mixed with 625 µl 0.5M $CaCl_2$ and 625 µl H_2O. Mixture was added dropwise to 1250 µl 2x HBS on a vortexer, incubated for 30 mins at RT and added to cells. Medium was changed the next morning and supernatant harvested after 48h. Lentivirus was concentrated by centrifugation (25000g for 90 min), resuspended overnight in 200 µl E8 and snap frozen in liquid nitrogen. 0.2 µl lentiviral solution were used per 24-well. Lentivirus was produced from the following plasmids: TetO-Ngn2-P2A-puromycin, rtTA, TetO-EGFP, and synapsin-NLS-mCherry. All plasmids were a generous gift from Prof. Thomas C. Südhof, Stanford University.

ROS assay

To increase cellular stress, iN cells were cultured in the absence of B27 for 3 days prior to the experiment. On DIV21 cells were treated with 5 µM CellRox green reagent (ThermoFisher) for 30 min at 37°C prior to fixation. NeuN staining was carried out as described below and coverslips imaged within 24h. Fluorescent intensity was analyzed by CellProfiler Software (Carpenter et al., 2006) by measuring 488 nm wavelength intensity in NeuN positive puncta (647 nm).

Analysis of DNA damage

Medium was changed two days prior to assay. On day 21, positive control was treated with 5 μM Etoposide (Sigma) for 1h, prior to fixation. NeuN and p-γ-H2AX staining was carried out as described below. Fluorescent intensity was analyzed by CellProfiler Software (Carpenter et al., 2006) by measuring 488 nm wavelength intensity in NeuN positive puncta (647 nm).

Immunofluorescence Experiments

IN cells were fixed in 4% paraformaldehyde in PBS for 20 min at RT. Cells were washed 3x for 10 min with PBS and incubated for 1h in blocking buffer (10% donkey serum, 0.1 % Triton X-100 in PBS). Primary antibody was incubated overnight at 4°C in staining buffer (PBS with 3% goat serum and 0.1 % Triton X-100). Subsequently cells were washed 3x with PBS and secondary antibody applied in staining buffer for 1.5h at RT. Cells were washed once and incubated with DAPI for 20 min, then washed 3x and mounted on cover slides. Immunofluorescence was visualized on a Leica SP8 confocal microscope.

Antibodies used:

Primary AB Name	Producer	Cat. No.	Dilution
Anti-Nanog	R&D	Af1997	1:100
Anti-TRA-1-60	Millipore	MAB4360	1:100
Anti-NeuN	Millipore	MAB377	1:100
Anti-Synapsin	Synaptic Systems	106001	1:500
Anti-Map2	Synaptic Systems	188003	1:600
Anti-γ-H2AX	Abcam	ab11174	1:500
Secondary AB Name	**Producer**	**Cat. No.**	**Dilution**
Dk-α-ms-Alexa488	Jackson	715-545-151	1:250
Dk-α-rb-Alexa488	Jackson	711-545-152	1:250
Dk-α-gt-cy3	Jackson	705-165-147	1:250
Dk-α-ms-Alexa647	Jackson	715-606-151	1:250
Dk- α-rb-Alexa647	Jackson	711-605-152	1:250

Immunoblotting and protein quantification

Cells were harvested in RIPA buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP40, 0.5% SDS, 0.5% sodium deoxycholate, 2 mM EDTA) supplemented with protease inhibitor (Roche) and phosphatase inhibitor cocktail 1 and 3 (Sigma)), snap frozen in liquid nitrogen and stored at -80°C until use. Lysates were analyzed by SDS-Page on 10 – 20% Tris-Tricine gels (ThermoFisher Scientific). Immunoblotting was performed using peroxidase conjugated antibodies and detected with an ImageQuant LAS 4000 system (GE Healthcare). Image analysis was carried out in ImageJ. Signals were normalized to β-III-tubulin probed on the same blot. Antibodies used:

Primary AB Name	Producer	Cat. No.	Dilution
Anti-APP (Y188)	Abcam	ab32136	1:1000
Anti-β-III-tubulin	Sigma	T8660	1:500
Anti-Drebrin	Cell Signaling	12243	1:1000
Anti-Drp1 (D6C7)	Cell Signaling	8570S	1:1000
Anti-Phospho-Drp1	Cell Signaling	3455S	1:1000
Anti-Mfn1	Cell Signaling	13196S	1:1000
Anti-Mfn2 (D1E9)	Cell Signaling	11925S	1:1000
Anti-Phospho-Tau (AT8)	ThermoFisher	MN1020	1:500
Anti-PSD-95	Millipore	CP35	1:400
Anti-Synapsin	Synaptic Systems	106001	1:1000
Anti-Tau	Synaptic Systems	314004	1:1000
Anti-Total OXPHOS WB Antibody Cocktail	Abcam	ab110413	1:500
Secondary AB Name	**Producer**	**Cat. No.**	**Dilution**
Anti-Mouse ECL IgG	GE Healthcare	NA931-1ML	1:4000
Anti-Rabbit ECL IgG	GE Healthcare	NA934V	1:4000
Anti-Guinea Pig IgG	Jackson	106-035-003	1:4000

Gene expression analysis

Quantitative RT-PCR was performed to determine mRNA levels of genes of interest. Total RNA was isolated by TRI-Reagent (Sigma). Technical triplicates of each experimental condition were pooled and RNA isolation was carried out according to manufacturer's instructions. DNA was digested by DNaseI (Fermentas). 1µg RNA was reverse-transcribed to cDNA using the iScript cDNA-synthesis kit (BioRad), according to manual. TaqMan probes with TaqMan Gene Expression Master Mix (ThermoFisher Scientific) were used for expression analysis on a 7900HT Real-Time PCR System (ThermoFisher Scientific). Tubb3 was used as an endogenous control. The assay ID of used TaqMan probes was: human Tubb3, Hs00801390_s1; human GAPDH, Hs02786624_g1; human Fis1, Hs00211420_m1; human Opa1, Hs01047018_m1.

MSD

Medium of iN cells was conditioned for 7 days and used for analysis. Human Aβ38/40/42 was analyzed with the Aβ Peptide Panel 1 (6E10) Kit (Meso Scale Discovery), according to manufacturer's instructions. Individual values were normalized to neuron numbers determined by dot blot assays probed against β-III-tubulin.

Karyotyping

Cells were arrested in metaphase by treatment with 80 ng/ml colcemide for 1h. Harvested and treated with hypotonic solution (0.075M KCl), followed addition of fix solution (MeOH 3:1 Acetic Acid). Metaphases spreads were stained by Quinacrine (Sigma), imaged on a Zeiss Axioskop HBO 50 fluorescent microscope (Zeiss) and arranged in Ikaros Software (MetaSystems).

Quantification and statistical analysis

Quantitative data represents means ± SEM. All experiments were independently repeated at least three times (with the exception of the ELISA assay which has been repeated twice). Statistical analyses were conducted with by Prism (GraphPad Software). Statistical comparison between multiple groups were analyzed by d'Agostino-Pearson omnibus normality test and subsequent one-way ANOVA with Tukey's test for normal distributed date or Kruskal-Wallis test with Dunn's multiple comparison post hoc test for non-normal distributed data. Statistical significance was only highlighted between control and patient-derived cells.

Ethics approval

All performed experiments were conducted with the approval and according to the regulations of the Zurich Cantonal Ethics Committee. Dermal punch biopsies were taken following informed consent at the Clinic for Dermatology of the University Hospital Zurich.

Conflict of interest

The authors declare no conflict of intrest.

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3.7 Figures and Tables

Table 1 Overview of recruited subjects for this study. Mean age at biopsy was 71.5 (HCS) and 74 (AD) with a range from 63 to 87 years. The individual age is not listed to avoid possible identification of the individuals as requested by the local ethics committee. n.d.: not determined.

Sample Name	Diagnose	ApoE	PiB-PET SUVR	Biomarker	Gender
HCS1	Healthy	3/3	1.07	-	Male
HCS2	Healthy	3/4	1.17	-	Male
AD1	AD	3/3	n.d.	Aβ: 550 pg/ml (576-1012); Tau-P: 162 pg/ml (<61); Total Tau: >1200 pg/ml (<500)	Female
AD2	AD	3/4	n.d.	Aβ: 201 pg/ml (576-1012); Tau-P: 160 pg/ml (<61); Total Tau: 1094 pg/ml (<500)	Female
AD3	MCI	3/4	2.4	-	Female
AD4	MCI	3/4	2.16	-	Female
AD5	AD	3/3	2.42	-	Female

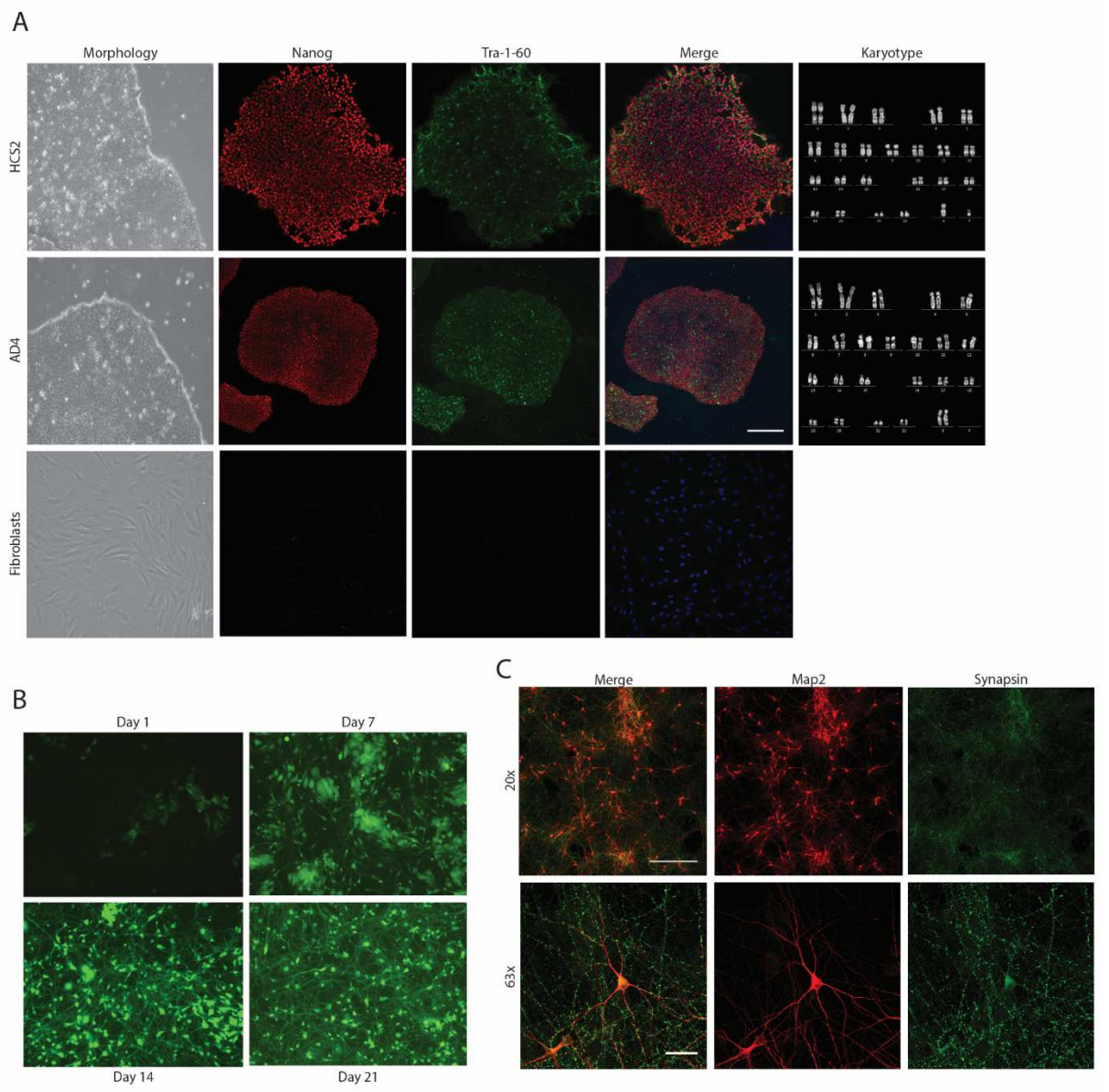

Figure 1 Fibroblast reprogramming to iPSCs and differentiation to iN cells.

A Representative images of one control and one AD patient-derived iPSC line. IPSCs but not fibroblasts showed an embryonic stem cell-like morphology (DIC) and expressed the pluripotency markers Nanog (red) and Tra-1-60 (green). Reprogrammed cells retained a normal karyotype. Scale bar: 300 µm.

B Differentiation of iPSCs to iN cells. Overexpression of Ngn2 led to induction of neuronal-like morphology over the course of 21 days, visualized by EPFG expression. Images were taken at 10x. **C** iN cells at day 21 stained positive for the neuronal marker Map2 (red) and the presynaptic marker synapsin (green). Scale bar: 200 µm (top), 25 µm (bottom).

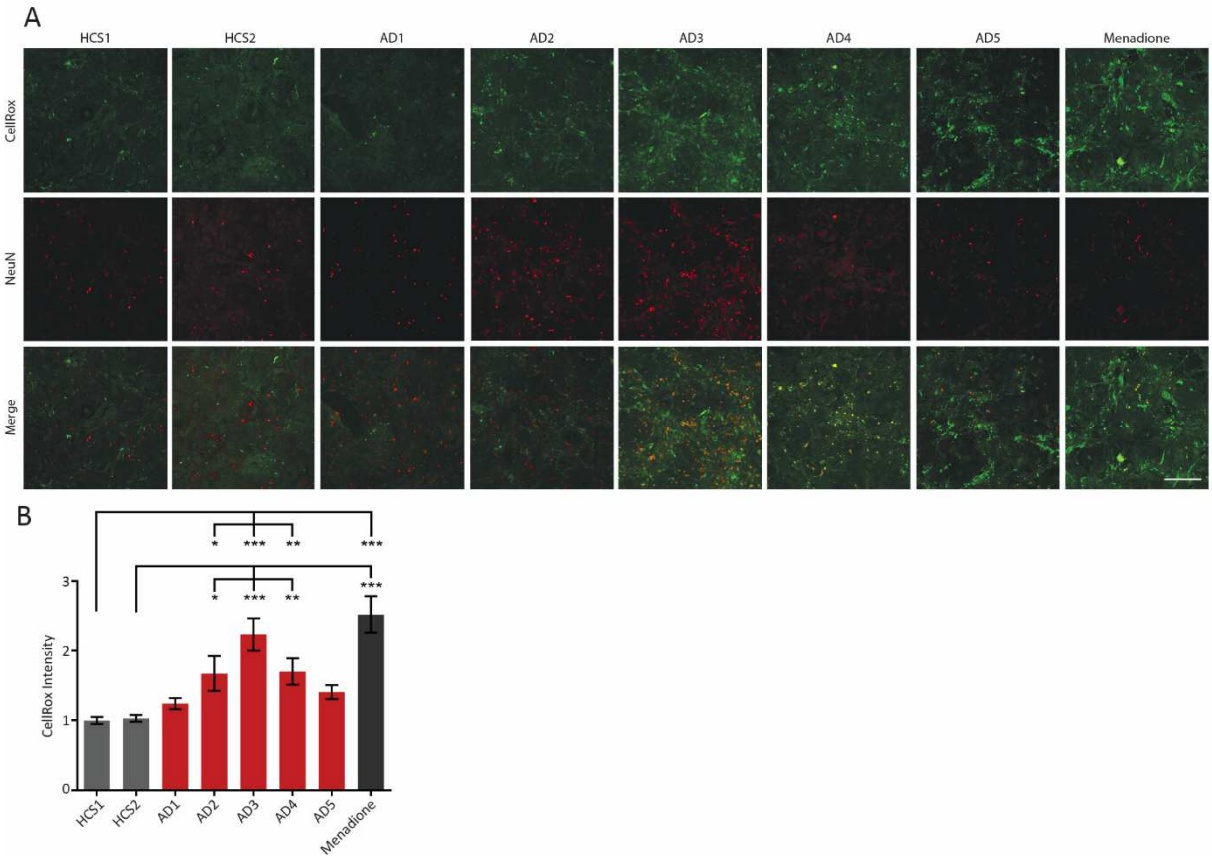

Figure 2 ROS production in AD patient and HCS iN cells. A Representative confocal images (20x) of iN cells stained with CellRox green dye followed by immunostaining against NeuN. CellRox intensity was assessed in overlay with NeuN positive puncta using Cell Profiler software. Menadione 50 µM was used as positive control. **B** Quantification of CellRox intensity. ROS production was significantly higher in AD2, AD3 ,and AD4 iN cells compared to HCS1 and HCS2. Scale bar: 200 µm. Data are means +/- SEM (n = 14 – 22). Statistical significance was assessed by Kruskal-Wallis test (*p<0.05; **p<0.01; ***p<0.001)**.**

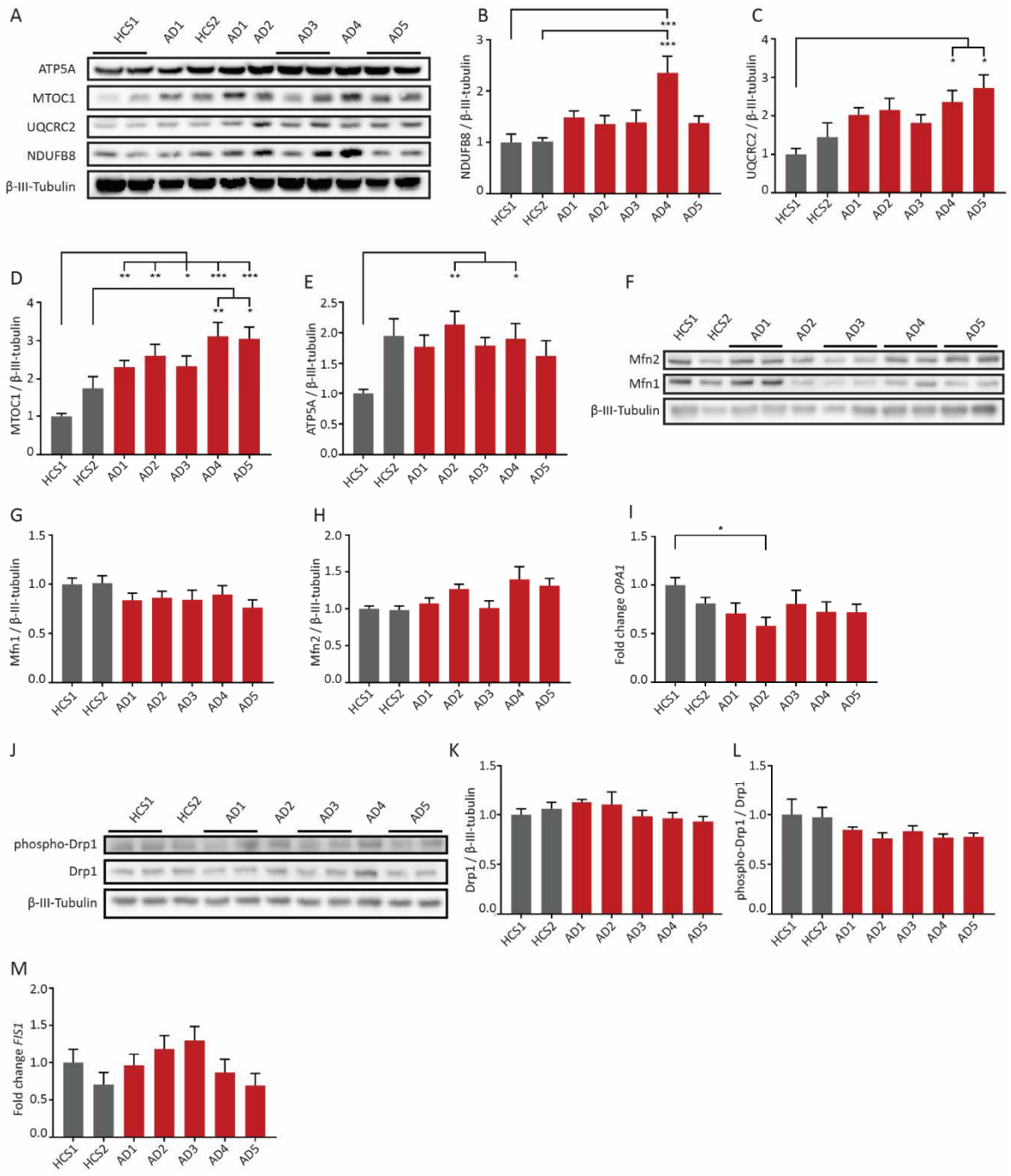

Figure 3 Levels of mitochondrial proteins and mRNA in iN cells from HCS's and AD patients. **A** Assessment of mitochondrial oxidative phosphorylation chain complex I, III, IV and V subunits by western blot using oxphos antibody cocktail. **B** Complex I subunit NDUFB8 was elevated in AD4 iN cells. **C** Complex III subunit UQCRC2 was significantly higher in AD4 and AD5 iN cells compared to HCS1. **D** MTOC1, a complex IV subunit, was higher in all AD-patient-derived iN cells compared to HCS1 and higher in AD4 and AD5 cells to HCS2. **E** Complex V subunit ATP5A levels were higher in AD2 and AD4 compared to HCS1. **F** Mitochondrial fusion proteins Mfn1 and Mfn2 levels were assessed by western blot. **G** Quantification of Mfn1 protein levels and **H** Mfn2 levels, both show no differences between HCS and AD-derived iN cells. **I** Level of *OPA1* mRNA, assessed by qRT-PCR, was lower in AD2 compared to HCS1 iN cells. **J** Fission protein Drp1 and phosphorylated-Drp1 levels were assessed by western blot. **K** Quantification showed no changes between HCS and AD-derived iN cells for total Drp1. **L** No changes were observed in phosphorylated-Drp1 levels to total Drp1. **M**

Levels of FIS1 mRNA were assessed by RT-qPCR and showed no difference between HCS and AD patient-derived iN cells. Data are means +/- SEM (n = 8 – 12). Statistical significance was assessed one-way ANOVA test (G-I; K-M) or by Kruskal-Wallis test (B-E) (*p<0.05; **p<0.01; ***p<0.001).

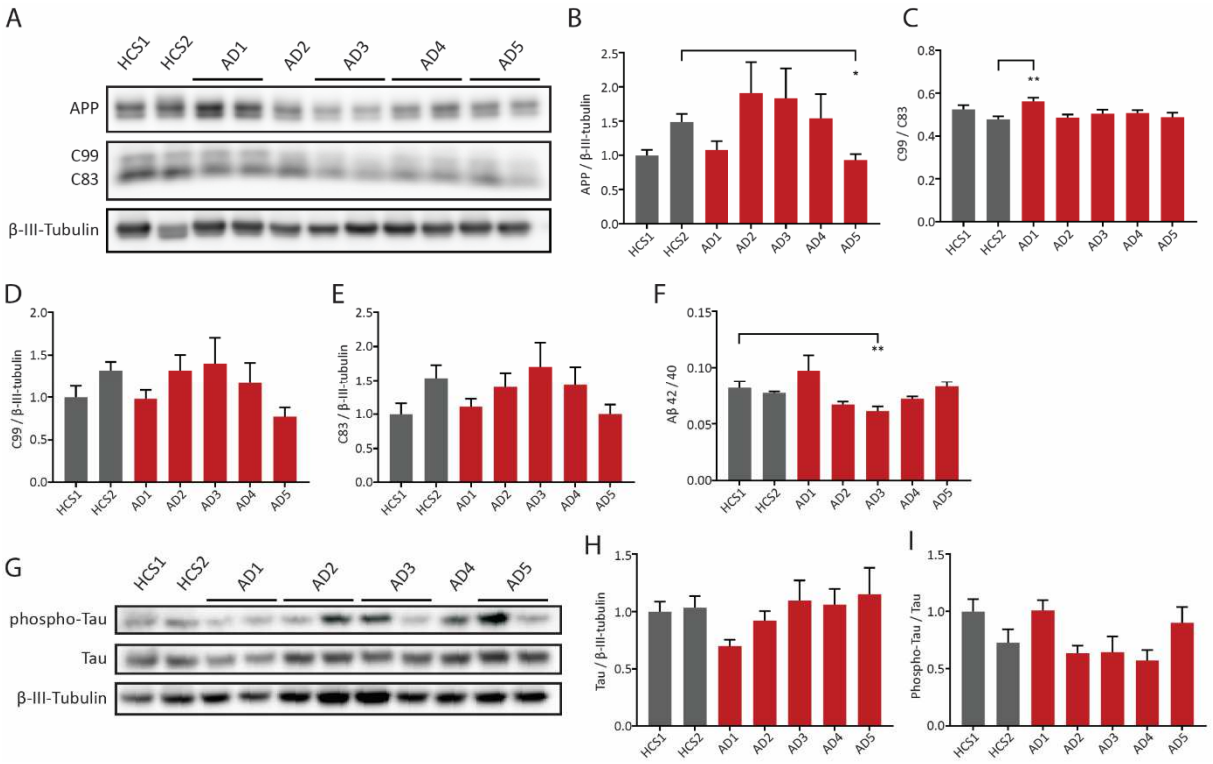

Figure 4 Characterization of AD hallmark proteins in HCS and AD patient-derived iN cells. **A** Protein levels of APP and α- and ß-secretase processing products C83 and C99, determined by western-blot. **B** Quantification of APP levels. HCS2 APP levels were significantly higher compared to AD5. **C** Quantification of C99 to C83 ratios. Ratio was shifted towards C99 in AD1 compared to HCS2. **D,E** Quantification of absolute C99 and C83 levels with no difference in AD and HCS-derived cells. n = 12 – 16; three independent experiments. **F** Analysis of Aβ42 to Aβ40 secretion of iN cells. Supernatant was conditioned from d21 to d28 and Aβ levels were subsequently measured by MSD. Aβ42 to 40 ratio is lower in AD3 compared to HCS1. **G** Assessment of total and phosphorylated tau levels by western blot. **H** Quantification of total tau levels in patient and control-derived iN cells. **I** Quantification of phosphorylated tau to total tau levels. **I,J** n = 12 – 18; Data are means +/- SEM. Statistical significance was assessed by Kruskal-Wallis test (*p<0.05; **p<0.01).

3.8 Supplemental Figures

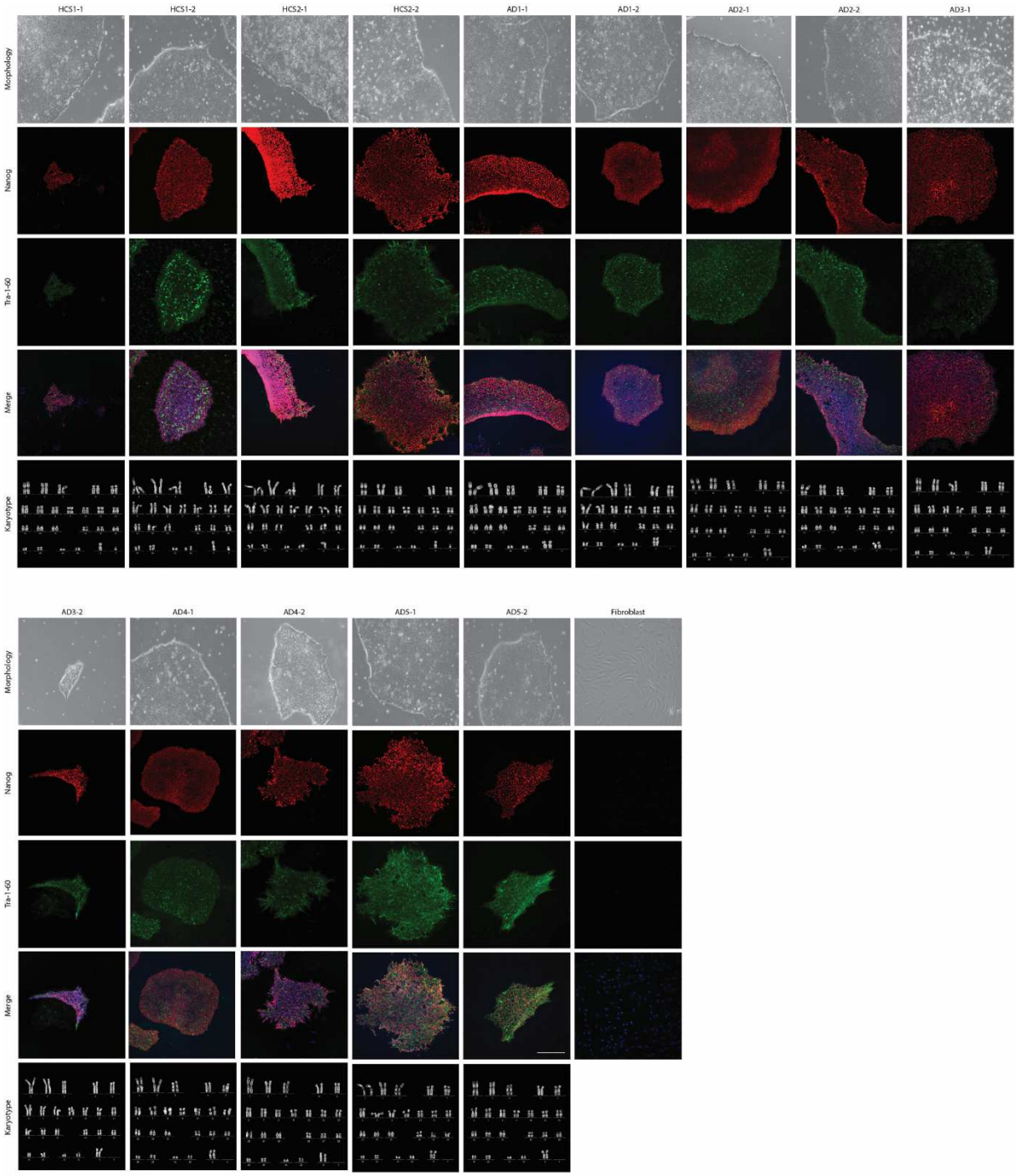

Figure S1 Characterization of all iPSC clonal lines used in this study, related to Figure 1. Morphology of iPSC colonies imaged in DIC (upper rows). iPSC colonies but not fibroblasts stain positive for Nanog (red) and Tra-1-60 (green) (rows 2-4), merged images also show DAPI (blue). Karyotype of iPSCs is normal after reprogramming (lower rows). Scale bar: 200 μm.

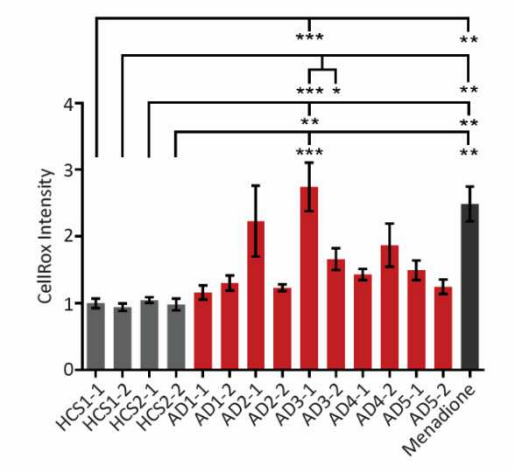

Figure S2 Quantification of CellRox intensities in individual clones, related to Figure 2. ROS production was significantly higher in AD3-1 and AD3-2 to the indicated HCS clones. Data are means +/- SEM (n = 7 – 11). Statistical significance was assessed by Kruskal-Wallis test (*p<0.05; **p<0.01; ***p<0.001)**.**

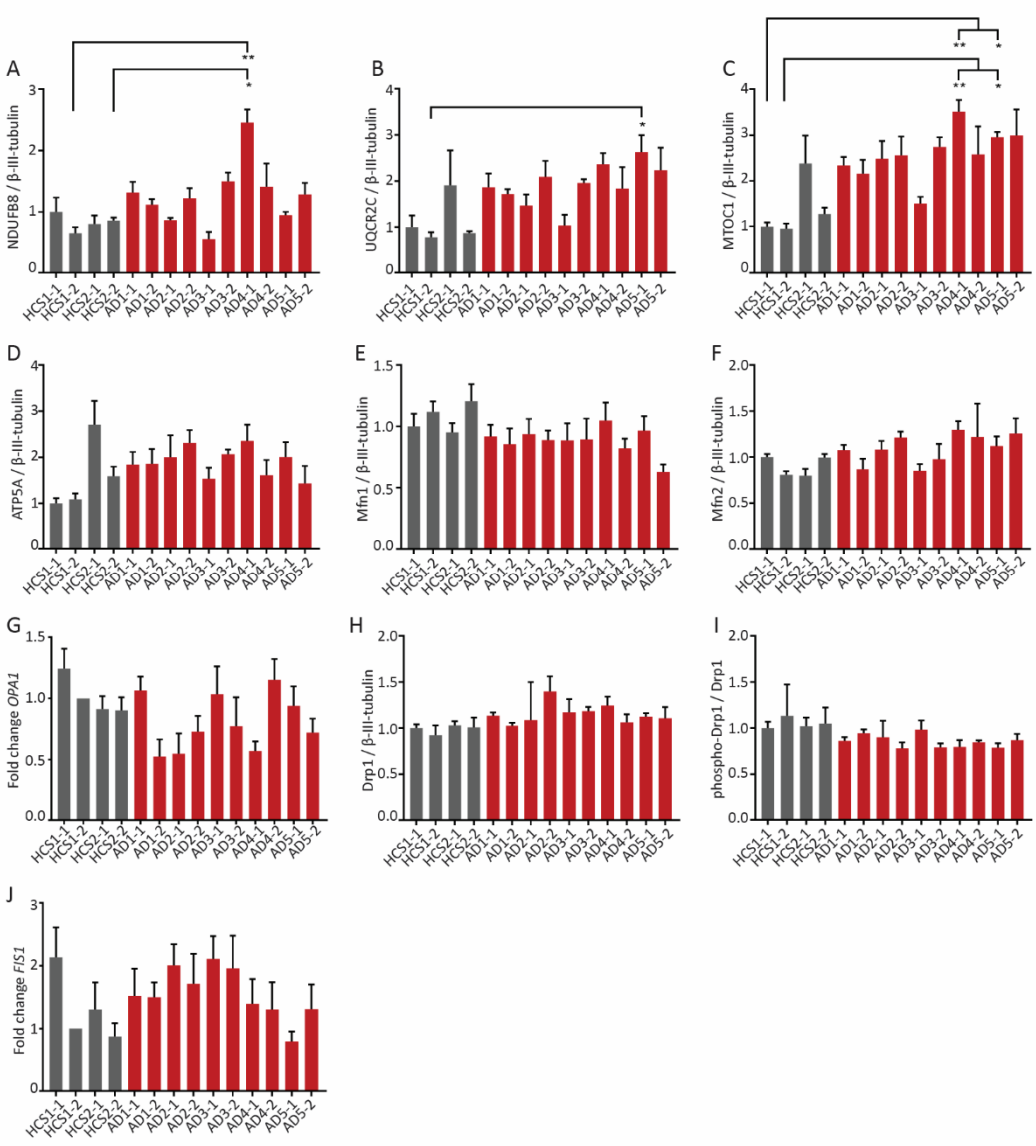

Figure S3 Protein/mRNA levels of mitochondrial proteins, shown as individual clones, related to Figure 3.

A Protein levels of mitochondrial complex I subunit Nudfb8 were higher in clone AD4-1 compared to control HCS1-2 and HCS2-2 clones. **B** Complex III subunit UQCRC2 protein levels were higher in clone AD5-1 than HCS1-2. **C** Complex IV subunit MTOC1 protein levels were higher in clone AD4-1 and AD5-1 compared to HCS1-1 and HCS1-2. **D** Protein levels of complex V subunit ATP5A were similar in individual patients and control clones. **E** Mfn1 protein levels showed no difference between clones. **F** Mfn2 protein levels were comparable in AD-patients and controls. **G** Opa1 mRNA levels were similar in HCS and AD patient-derived cells. **H** Levels of Drp1 protein displayed no difference between HCS and AD cells. **I** Ratio of phosphorylated Drp1 to total Drp1 protein was comparable in individual clones. **J** No changes in Fis1 mRNA levels, determined by qRT-PCR were detected. **G,J** Fold change mRNA levels were determined by the ΔΔCT method with clones being normalized to clone HCS1-1. Data are means ± SEM. For statistical analyses, Gaussian distribution of data was determined using d'Agostino-Pearson omnibus normality test. Data are means +/- SEM (n = 4–6). For comparison of clonal lines one-way ANOVA or Kruskal-Wallis test was used (*p<0.05; **p<0.01; ***p<0.001).

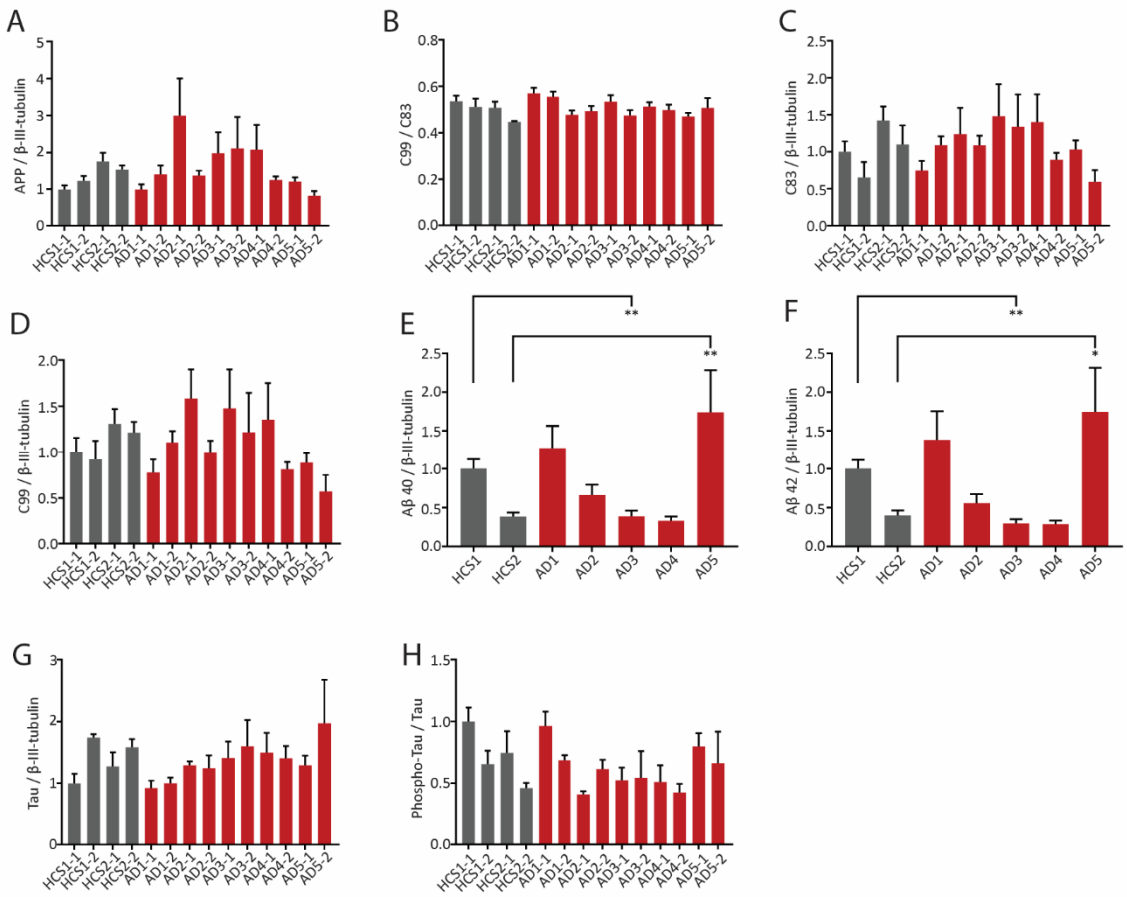

Figure S4 APP levels and processing in HCS and AD patient-derived iN cells, related to Figure 4. A No changes were observed in APP protein levels in AD patients and HCS-derived iN cells, determined by western blot. **B** C99 to C83 ratio was not altered in clones from HCS's and AD patients. **C,D** Equal C83 and C99 protein levels in iN cells clones from AD patients and HCS's. **E,F** ELISA showing higher Aβ40 and 42 levels in HCS1 compared to AD3 and lower in HCS2 compared to AD5. **G,H** Tau and phosphorylated Tau levels did not show changes in individual clones. Data are means ± SEM (n=6–9). Statistical significance was assessed by one-way ANOVA (*p<0.05; **p<0.01).

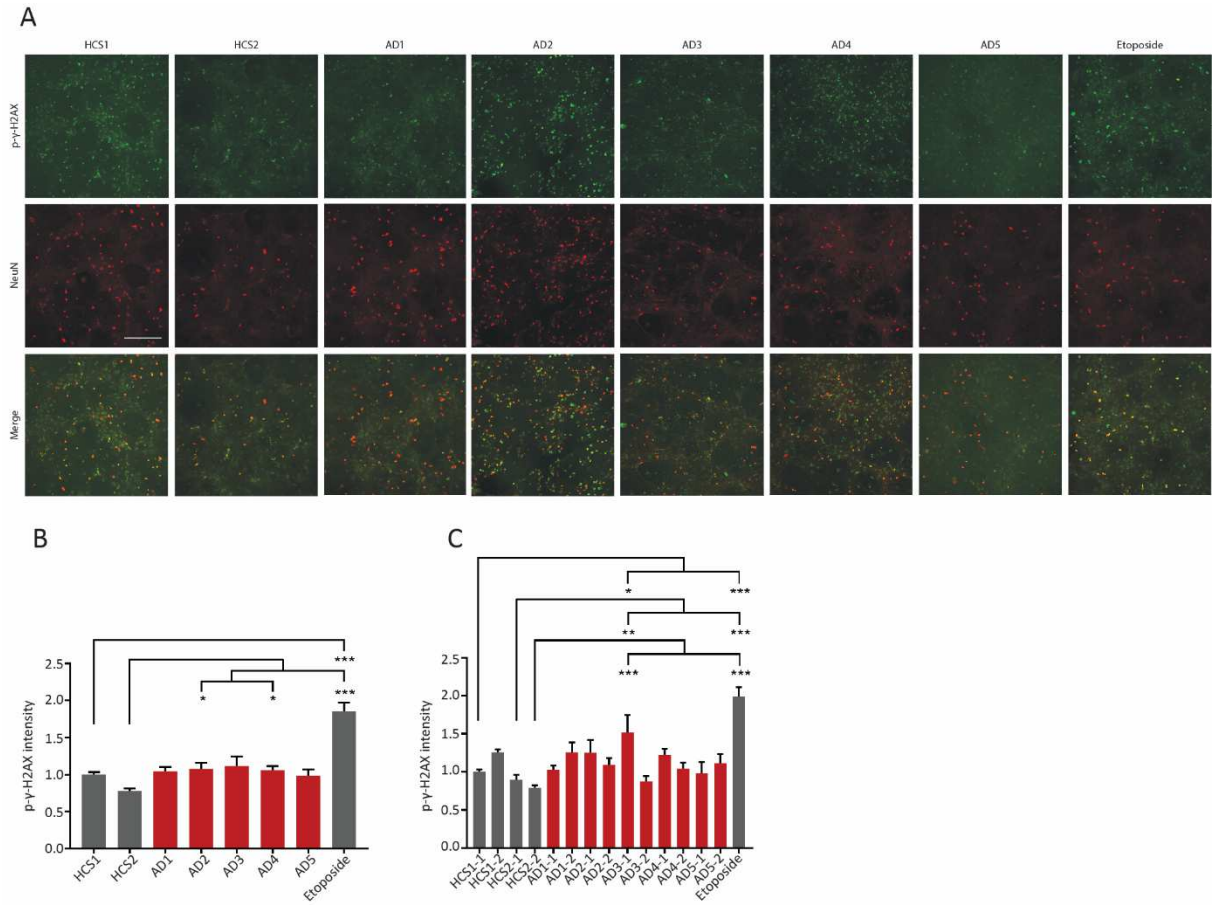

Figure S5 DNA damage in HCS and AD patient-derived iN cells

A Representative images of phosphorylated γ-H2AX intensities (green), as a readout of DNA damage, in AD patient and HCS-derived iN cells. NeuN (red) was stained to identify neuronal nuclei. Intensities of phosphorylated γ-H2AX were measured in NeuN positive puncta in an automated fashion using Cell Profiler software. Cells treated with 5 µM Etoposide for 1h were used as a positive control. **B** Quantification of phosphorylated γ-H2AX intensity showed higher intensities in AD2 and AD4 compared to HCS2. **C** Quantification of individual clones of patients and HCS. Clone AD3-1 showed higher signal intensities than controls HCS1-1, HCS2-1 and HCS2-2. Data are means ± SEM. Statistical significance was assessed by one-way ANOVA or Kruskal-Wallis test (*p<0.05; **p<0.01; ***p<0.001).

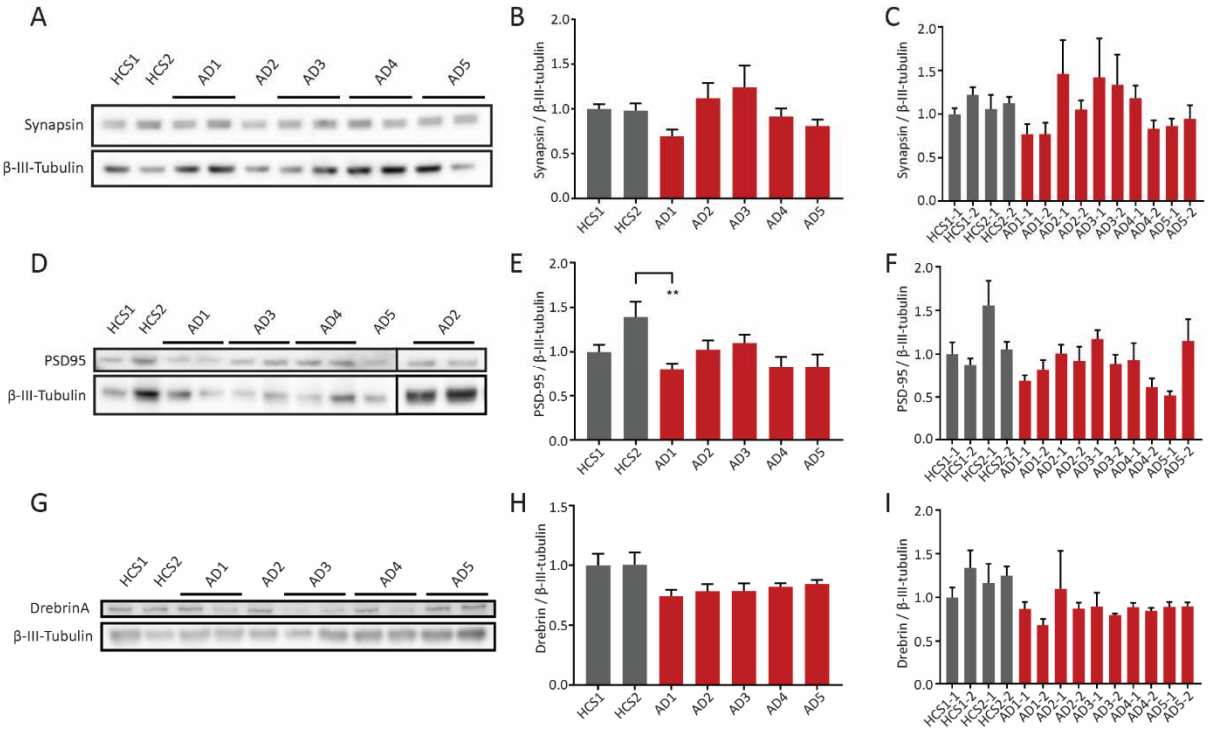

Figure S6 Synaptic protein levels in AD patient and HCS-derived iN cells

A Levels of presynaptic synapsin1 were assessed by western blot. **B** Quantification did not show differences between iN cells from the five patients to two controls. **C** No differences between iN cells from individual clones were observed **D** Western Blot of PSD-95 protein levels. **E** Protein levels of post-synaptic protein PSD-95 showed lower levels in AD1 compared to HCS2. **F** Protein levels of individual clones from each patient and HCS did not show significant changes in PSD-95. **G** Protein levels of the post-synaptic protein drebrin, determined by western blot. **H** Drebrin shows no significant changes between HCS and AD-derived cells. **I** Individual patient and control clones showed no difference in drebrin levels in AD patient-derived iN cells.

Data are means ± SEM (n=6–9). Statistical significance was assessed by one-way ANOVA or Kruskal-Wallis test (*p<0.05; **p<0.01).

Chapter IV

Discussion and Outlook

4.1 Metabotropic function of NMDARs

4.1.1 Aβ-mediated synaptic toxicity

We have shown that Aβ mediates spine loss and LTD induction is independent of ion flux. This mechanism was proven in both hippocampal slices of arcAβ transgenic mice and in wild type hippocampal slices treated with Aβ. Aβ exists in a multitude of aggregation states from monomer to fibril. We did evaluate the composition using SDS PAGE and showed a mixture of mainly monomers to tetramers but SDS was shown to disrupt oligomers and thus might not reflect the actual composition (Benilova et al., 2012). Validation of Aβ composition would require further analysis by size exclusion chromatography or atomic force microscopy. Synapses are thought to be the central unit of memory formation and storage. The onset of pathological symptoms with memory decline and synapse loss (Terry et al., 1991) could be induced by Aβ binding to NMDARs and lead to activation of LTD. This would be in line with APP being localized at synapses and produced Aβ acting locally on surrounding cellular structures (Schubert et al., 1991). Microglia-mediated aberrant synaptic loss has recently been proposed as an alternative mechanism to LTD mediated synapse loss (Hong et al., 2016), yet it is possible (and likely) that both systems contribute to memory decline. However, it is not understood when Aβ mediated synaptic depression arises and which local concentrations have to be reached for this effect to start. Diverse targets of Aβ have been identified, such as NMDARs, AMPARs, EphA4, and mGluR5, which are expressed in different cell types of the brain (de Strooper and Karran, 2016). So far, it was not possible to delineate different mechanisms of Aβ-induced toxicity in order to determine preferential binding partners of Aβ and define their contribution to overall Aβ-mediated toxicity. Further, neuronal cells have been identified as most susceptible cell type in AD and their loss leads to brain atrophy, yet this process is likely to be influenced by effects of astrocytes, oligodendrocytes and microglia (de Strooper and Karran, 2016). Though pathological changes in these cells have been identified, limited understanding has been achieved in how these affect other cell types and the complex system of the brain.

4.1.2 Emerging and contradicting functions of the NMDAR

It has been a long standing paradigm that Ca^{2+} signaling mediates LTD and could be prevented by use intracellular Ca^{2+} chelators, such as BAPTA (Collingridge et al., 2010; Dudek and Bear, 1992; Mulkey and Malenka, 1992). Further, LTD was found to depend on signaling via calcineurin and hippocalcin, which require Ca^{2+} for their activation. Yet, recent lines of research emerged that showed LTD was independent of Ca^{2+} influx (Nabavi et al., 2013). Intracellular application of BAPTA bound additional and possibly influxing free Ca^{2+} with baseline Ca^{2+} sufficient to lead to LTD. Moreover application of MK-801, an open channel blocker, and 7CK, a GluN1 antagonist, could not prevent LTD, whereas treatment with APV, a GluN2 blocker prevented LTD. Further studies confirmed these findings (Kessels et al., 2013; Tamburri et al., 2013) and we extended these by showing that also Aβ induced

synaptic loss was independent of Ca^{2+} influx and required p38-MAPK activation (Birnbaum et al., 2015). Moreover, hereafter published evidence confirmed our findings that spine shrinkage was dependent on NMDAR metabotropic signaling using 2-photon glutamate uncaging in the presence of 7CK and MK-801 (Stein et al., 2015). Further research showed that metabotropic signaling affects the interaction between NMDAR CTD and CaMKII, dependent on PP1 (Aow et al., 2015) and that LTD is mediated by postsynaptic NMDARs (Carter and Jahr, 2016). Excessive NMDAR activation can lead to cytotoxicity and was thought to depend on Ca^{2+} influx (Rossi et al., 2000; Zhang et al., 2016). Yet, it was shown that also metabotropic NMDAR signaling conferred toxicity, independent of Ca^{2+}. Activation of NMDAR led to complex formation with Src kinase and Panx1 and blockage of complex formation prevented excitotoxicity. Further, this showed metabotropic NMDAR function for the first time *in vivo*, whereas prior experiments were carried out in slice cultures (Dore et al., 2016; Weilinger et al., 2016). Yet, how can the discrepancy between the initial 20 years of research, with Ca^{2+} dependent LTD (Mulkey and Malenka, 1992) and the newly emerging line of research, describing a metabotropic function of the NMDAR with Ca^{2+} independent LTD be explained? A contradicting report to the initial publication of metabotropic NMDAR signaling-mediated LTD showed Ca^{2+} influx was necessary for LTD induction. MK-801 application also prevented p38-MAPK activation and dephosphorylation of AMPA GluR1 subunit (Babiec et al., 2014), with another study also reporting ablation of LTD in the presence of MK-801 (Sanderson et al., 2016). The origin of these differences is not clear. One explanation could be that both pathways, ion flux independent and dependent LTD coexist. Variations in sample preparation and treatment could lead to induction of one of both. Other possibilities include the chemicals used in the various studies. 7-CK which is an antagonist of the glycine binding site on GluN1 showed affinity to other ionotropic receptors (Foster et al., 1992) and all studies entirely relied on AP5 for inhibition of the glutamate binding site on GluN2. However, the cause remains elusive and further investigation will be required.

4.2 Induced pluripotent stem cells

4.2.1 AD-related pathology in patient-derived induced neurons

Aberrant mitochondrial function has been reported frequently in AD (DuBoff et al., 2013; Grimm et al., 2015). We detected higher levels of ROS in iN cells from three of five patients. This is in line with prior findings that showed increased ROS levels in 12 month old triple transgenic AD mice (overexpressing APP Swedish, MAPT P301L and PSEN1 M146V), APP/PS2 mice (overexpressing APP swedish and PSEN2 N141I mutations) (Rhein et al., 2009), and higher levels of oxidative damage in Tg2576 mice, overexpressing APP Swedish (Reddy et al., 2004). Further, increased ROS levels were detected in iPSC-derived neuronal cells of patients with an APP E693Δ mutation and in iPSC-neurons from one out of two sporadic AD patients (Kondo et al., 2013). Moreover, postmortem

brain tissue from AD patients showed high oxidative damage (Mecocci et al., 1994). Thus we can assume that increased oxidative stress represents a common feature of AD, yet in mouse models and postmortem tissue it was difficult to assess the onset of higher stress levels. Neuronal cell derived from iPSCs display an age signature of young cells, thus we speculate that this phenotype arises early in AD. However, we cannot fully exclude that the observed increase in stress levels were due to mutations which occurred in donor fibroblasts and are unrelated to AD. Further, fibroblasts with increased ROS levels could be preferentially reprogrammed. IPSCs from aged donors were found to show increased ROS scavenging activity due to low levels of Zscan10, which negatively affected genomic stability by abnormal ROS-glutathione homeostasis (Skamagki et al., 2017). Thus, higher ROS levels would increase their genomic stability. Mitochondria in mitotic cells are thought to have a lower mutational load than mitochondria in long-lived postmitotic cells and a lower threshold to undergo apoptosis in case of excessive damage, as mitotic cells can be replaced (Terman et al., 2010). Consequently, we further cannot rule out that our iPSC-derived neuronal cells underestimate the actual level of mitochondrial dysfunction. In line with increased ROS production, we also witnessed increased levels on DNA damage in the same cell lines which were affected by ROS production, compared to AD2. DNA damage and higher ROS levels were described to have a reciprocal additive effect (Quirós et al., 2016).

We further analyzed the composition of the mitochondrial oxidative phosphorylation chain, with complex I and III of the respiratory chain being the main sites of ROS production. We found an upregulation of complex IV subunit MTOC1 in all and upregulations of complex I, III and V in certain AD-derived iN cells. Cells from patient AD4 showed higher levels of complex I and III, which could explain the increased ROS levels, yet in AD2 and AD3 patient-derived iN cells no changes in these complexes were observed, thus ROS production here might be caused by an alternative mechanism, with complex IV upregulation as part of an oxidant defense mechanism (Turrens, 2003). The changes in oxphos protein levels were in line with reports showing an upregulation in complex I, III, IV and V in brains of AD patients (Nagy et al., 1999) and Tg2576 mice (Reddy et al., 2004), whereas contradicting reports found lower levels of COX1 in complex IV (Bosetti et al., 2002; Pérez-Gracia et al., 2008). We speculate that the upregulation of oxphos complexes could represent an initial compensatory mechanism to cope with reduced ATP production and later in disease a breakdown of the oxphos chain leads to neuronal death. Ultimately the question is which changes arise first, could defects in mitochondria set off the detrimental effects of AD or are changes in mitochondria a consequence of the effects of AD pathology. Assuming a synergistic effect, this may lead to a vicious circle of pathology. Mitochondrial mutations which accumulate during the process of normal ageing could lead to a higher generation of ROS, which has been shown to increase Aβ levels (Leuner et al., 2012). Increased Aβ would lead to mitochondrial malfunctioning and thus further increase ROS generation. Likewise, mitochondrial defects could arise from Aβ-induced dysfunction and lead to increased ROS production. Though the first mentioned could influence the disease, it is important to

note the lack of mitochondria-related risk genes in AD and that mitochondrial mutations have been associated with other neurodegenerative disease such as PD. It has been reported that abnormal mitochondrial fission and fusion contributes to AD pathology (DuBoff et al., 2013) and reduced OPA1, MFN1, and MFN2 levels and higher FIS1 levels were found in human AD brain tissue (Wang et al., 2009). Contradicting results were found for Drp1 (Manczak and Reddy, 2012; Wang et al., 2009). We observed lower levels of Opa1 mRNA levels in only one of the patient-derived iN cells, whereas MFN1+2 and DRP levels were unaffected in all patients. Thus we conclude that fission and fusion were mostly intact in iN cells from AD patients, representing an early stage of the disease but might be more severely affected later in disease. During reprogramming mitochondrial age is reset and thus we cannot rule out that fission and fusion abnormalities arise during ageing and are exacerbated due to AD pathology.

Interestingly, we were not able to detect major changes in APP levels, Aβ42/40 ratios or C99/C83 levels. Cells from one AD patient displayed lower APP levels compared to one control and another patient cell line showed a lower Aβ42/40 ratio. Our observations are in line with prior observations that familial but not sporadic patients have higher APP levels or increased rates of Aβ production. Total Aβ40 and 42 levels were higher in iN cells of patient AD5 and could have contributed to disease pathology. Even though not significant against HCS1 we cannot exclude that this could be due to changes in APP copy number (Bushman et al., 2015). However, as total APP, C99 and C83 levels were comparable between all lines one explanation for higher Aβ 40 and 42 levels in the supernatant could be a stronger secretion of Aβ. Moreover, the increase in Aβ 40 and 42 levels did not correlate with increased ROS production.

The second hallmark protein of AD is tau and its aberrant phosphorylation and aggregation has been linked to neurodegeneration. In our study we could neither detect changes in total tau levels nor in phosphorylation at Ser202 or Thr205. This is in line with the idea that Aβ accumulation leads to tau phosphorylation as a downstream target. It is important to note that iN cells express 3R tau and no 4R tau, which reflects the embryonic situation, whereas in adult neurons both types are expressed at the same ratio (Sposito et al., 2015).

We further assessed if synaptic deficits are detectable in our iN cells, as synaptic loss has been demonstrated to correlate with cognitive decline (Terry et al., 1991). Examination of pre- and postsynaptic proteins, i.e. synapsin, drebrin and PSD-95, did not show differences between control and SAD patient-derived cells. Using iPSC-derived neurons we aimed to assess a presymptomatic, early phase of the disease, thus synaptic alterations were not expected, especially in regard that we and other groups linked synaptic loss to Aβ-mediated toxicity and we could not detected higher Aβ levels in iN cells from patients. In addition, we have shown that synaptic loss is mediated by ion-flux-independent metabotropic NMDAR signaling. However, neuronal cells induced by Ngn2 overexpression were shown to express low levels of NMDAR subunit GluN1, GluN2A and GluN2B (Zhang et al., 2013). Thus, it is likely that mechanisms of synaptic depression will not be exhibited in iN cells and not be

reflective of mature neuronal cells in the human brain. Induction of LTP and LTD have, to our best knowledge, not been shown in iPSC-derived neuronal cells, independent of the differentiation protocol. Further limitations of this study represent the small sample cohort and in this regard the use of male controls and female SAD patients. However, heterogeneity between our AD patients makes it unlikely that the examined differences are gender effects. Further, studies of mitochondria of male and female mice showed no difference in bioenergetics, oxidative stress and apoptosis (Sanz et al., 2007). Yet, the use of additional iPSC lines would be required to rule out that the observed effects represent sex differences. Meta-analysis of two large scale studies on variation of iPSCs (Carcamo-Orive et al., 2016; Kilpinen et al., 2017) led to the proposition that comparison of clones from unrelated individuals is more specific when only one clone per individual is used for analysis instead of two. Loss of sensitivity would be best accounted for by use of isogenic controls. Further, sensitivity reached a plateau when 6 subjects were used per group (Germain and Testa, 2017). This analysis referred to data obtained from cultured iPSCs, thus further studies would be necessary to prove if these claims hold true for differentiated cells, which likely represent a more heterogeneous population. Our study showed altered ROS production and mitochondrial complex composition in some of SAD patient-derived neuronal cells independent of Aβ and tau, which suggest a disease-initiating influence of mitochondrial dysfunction in an early, presymptomatic stage of AD. One possible mechanism could be that ROS and ETC complex dysregulation lead to a higher susceptibility towards Aβ insult.

4.2.2 Current challenges in the field of iPSCs

Induced pluripotent stem cells hold a great potential for drug screening, disease modeling, and cell therapy. Arguably, iPSCs changed the way of biomedical research and opened new venues for therapies. Despite all merits, iPSCs are, so far, limited in a number of ways for the above mentioned applications. These can be broadly classified in two fields, limitations due to the biology of iPSCs and for reasons of technical challenges. It has been observed that reprogrammed somatic cells vary from one clone to the other, leading to a molecular heterogeneity between iPSC lines. This variability has been subject of research and several sources were identified. Reprogramming PBMCs from donors with different ages showed an age influence on the mutational load, with higher age exonic mutations being more frequent (Sardo et al., 2016). Further it was found that cells from older donors retained a epigenetic memory of age, yet this effect could be diminished by passaging (Kim et al., 2011; Sardo et al., 2016). Residual DNA methylation patterns were found to depend on the reprogramming technique. Reprogramming by somatic nuclear transfer gave rise to stem cells which resembled ES cells derived from *in vitro* fertilization in their methylation profile, whereas iPS cells generated by ectopic OSKM expression displayed residual DNA methylation and an aberrant transcriptomic profile (Ma et al., 2014). Differential methylation was found to be enriched in regions with MYC binding motif and is clone-specific, independent of genetics (Panopoulos et al., 2017). Furthermore, a higher load of mitochondrial mutations was detected in fibroblast-derived iPSCs from elderly donors, showing both homoplasmic and heteroplasmic mutations. These were identified by sequencing of expanded iPSC

lines, but not detected by bulk sequencing of fibroblast samples, reflecting the occurrence of random somatic mutations in individual cells (Kang et al., 2016). The donor cell type has been identified as another source of variation of iPSCs, as cord blood cells and keratinocyte-derived iPSCs showed different methylation patterns which ultimately affected their differentiation potential (Kim et al., 2011). Reprogramming of somatic cell to pluripotency does involve reorganization of the epigenetic landscape but also induces genetic aberrations. Copy number variations (CNVs) in iPSCs were linked to a change in replication timing from somatic to stem cell. CNV gains were observed in early genome replication during reprogramming, whereas loss of CNVs occurred later in replication (Lu et al., 2014). Genetic variation was identified as a reason for heterogeneity of iPSC lines (Burrows et al., 2016; Rouhani et al., 2014). Most studies suffered from low sample numbers and thus limited power. Recently, three groups published large-scale studies addressing the origin of molecular heterogeneity in iPS cells (Carcamo-Orive et al., 2016; DeBoever et al., 2017; Kilpinen et al., 2017). The studies used 100 – 300 individuals to derive 215 – 711 iPSC lines for analysis and found that about 50% of iPSC heterogeneity between donors derives from genetic variation. This effect was stronger than any other factor, such as CNVs, passage, gender or culture conditions (Carcamo-Orive et al., 2016; Kilpinen et al., 2017). Further the three studies mapped expression quantitative trait loci (eQTLs) by identifying regions with DNA variation which affected mRNA expression levels and thereby attributed to the observed genetic variation. Moreover, Carcamo-Orive *et al*. showed that iPSC variability was also influenced by polycomb target genes by an eQTL-independent mechanism. Kilpinen *et al*. identified that 41% of 711 cell lines had CNVs, with most of them unique to an individual iPSC line. Further, CNVs were enriched in 35 genomic locations, with the three most frequent ones being trisomy of the X chromosome and duplications in chromosome 17 and 20. As depicted above, iPSCs are still not fully understood in regard to reprogramming-induced changes in the genome and epigenome and how this will affect differentiated cells, where the impact of genetic variation was found to be higher (Banovich et al., 2016). Technical challenges mostly concern culturing of iPS cells, which induces chromosomal aberrations, ranging from deletions, to duplications, to trisomy or to loss of chromosomes (International Stem Cell Initiative, 2011; Taapken et al., 2011). Generally these effects confer a growth advantage to affected cells. We cannot exclude that the changes we observed in our iPSC-derived iN cells were also affected by these iPSC culture-induced changes, with cells subjected to change over the course of passaging and genome instability (Lamm et al., 2015). Moreover, prolonged culturing of primed iPSCs leads to erosion of X-chromosome inactivation in female iPSCs and loss of silencing cannot be reset by differentiation, resulting in different gene expression patterns between early and late iPSCs (Mekhoubad et al., 2012; Papp and Plath, 2013). Freezing and thawing will also lead to variation within a population of donor cells, as a further point of culture induced variance (Wong et al., 2017). A recent study observed an enrichment in P53 mutations in iPS cells, a gene often mutated in cancer (Ghosh et al., 2017). This is of particular importance for future applications of cell replacement therapy, which raises concern

about safety and will require more stringent quality control.

4.2.3 Future directions

Our study revealed mitochondrial alterations in SAD patient-derived iN cells. However, the observed changes did not prove whether mitochondrial functionality was impaired and if so, to what extent. Thus, further analysis could include determination of mitochondrial membrane potential (Sompol et al., 2008), measurements of O_2 concentration and consumption rates and activity of different complexes. Moreover, assessment of ATP levels in mitochondria allows conclusions on functionality (Rhein et al., 2009). Further, upregulation of mitophagy would be indicative of malfunctioning mitochondria. We examined mRNA levels of SIRT1, ATM and other proteins involved in mitochondria maintenance and damage signaling but could not observe changes (data not shown) (Fang et al., 2016; Quirós et al., 2016). Mitochondrial morphology is thought to be indicative of mitochondrial dysfunction (Picard et al., 2013). We aimed to assess morphology by antibody staining, use of mitotracker and transfection of plasmid expressing a fluorescent tagged F1F0-ATP synthase, yet the obtained results showed strong variation within cells from one subject (data not shown). In order to identify dysregulations in pathways which could contribute to disease it would be advantageous to carry out RNA sequencing and thereby obtain information for the whole transcriptome. This allows unbiased analysis, whereas we and recent publications focused on proteins known to be altered in disease. In addition, proteome and metabolome analysis would complement this approach. In AD massive neuronal loss is observed, thus patient-derived iN cells could exhibit a higher susceptibility towards different stressors, ultimately leading to cell death. We assessed long-term survival of iN cells using expression of an NLS-mCherry construct under the synapsin promotor, yet imaging up to 113 days after induction led to inconsistent results across the iN cell lines and time. Addition of acute stressors, such as Aβ or H_2O_2, with consecutive LDH or apoptosis marker assays represent a more direct readout for examination of cell death.

An extension of this, and an in general interesting venue for experiments on diseases of age, is the induction of an age phenotype. Is the accumulation of Aβ over time sufficient to develop AD or are in addition normal age-related physiological changes required for disease onset? Progressive loss of RanBP17 over the course of ageing was identified in fibroblasts, cortical cells, kidney, thyroid carcinomas and glioblastomas from aged individuals. This led to breakdown of the nucleo-cytoplasmic compartmentalization which affected protein trafficking and localization (Mertens et al., 2015a). A similar mechanism of nucleo-cytoplasmic barrier breakdown could play a role in Hutchinson-Gilford-Syndrome in which expression of a mutant form of laminA (progerin), an integral protein of the nuclear lamina, leads to premature ageing. Overexpression of progerin was shown to induce some age-associated markers in iPSC-derived dopaminergic neurons (Miller et al., 2013). However, it remains to be shown how closely knock down of RanBP17 or progerin overexpression recapitulate hallmarks of physiological ageing in regard to accumulation of DNA damage, genome instability, telomere

attrition, loss of proteostasis, epigenetic alterations, and mitochondrial dysfunction.

We showed that the levels of pre- and postsynaptic proteins were not altered in SAD-derived iN cells. Transmission of information by neurotransmitter release leads to depolarization of the postsynapse, membrane potentials are propagated and summated at the axon where action potentials (APs) are evoked (Südhof, 2004). Thus further points of evaluation could be measurements of miniature excitatory postsynaptic potentials (mEPSCs) and evoked APs by electrophysiological methods. Electrochemical potentials can also be manipulated using light-inducible channels, allowing the rapid and specific control of neuronal activity (Boyden et al., 2005). Optogenetics allows precise control over ion-flux of Na^+, Cl^-, H^+ and Ca^{2+} (Kyung et al., 2015; Wang et al., 2017; Yizhar et al., 2011) and thus enables complex manipulation of neuronal circuits and cells *in vivo* and in *vitro* (Ramirez et al., 2013). It is of importance for the physiological function of neuronal circuits that signals are not only enhanced but also negative feedback is provided by inhibition. Thus electrophysiological properties of iN cells would represent a more physiological population by the addition of iPSC-derived GABAergic neurons (Yang et al., 2017a). It has further been shown that synaptic activity of iPSC-derived neurons is enhanced by use of serum-free medium and other neuroactive peptides (Bardy et al., 2015). However, use of defined cell types for assays, as we did by overexpression of Ngn2, leads to a reduced variability and thus allows identification of weak effects, which could be confounded in the presence of a heterogeneous neuronal population.

Our analysis is based on the comparison of non-related individuals, which makes it difficult to correlate observed effects. In order to study effects of mutations on cellular processes genome engineering allowed generation of isogenic cells, differing (in theory) only in the gene of interest (GOI). The first isogenic lines were created with zinc finger nucleases to obtain models for early onset PD mutations (Soldner et al., 2011). The discovery of Crispr-Cas9 based genome editing proved to be more versatile and efficient (Cong et al., 2013; Jinek et al., 2012). Here, Cas9 induces a DNA double strand break in the GOI, directed by a guide RNA. Cellular repair mechsnisms either utilizes non-homologous end joining (NHEJ) or homology directed repair (HDR). Whereas NHEJ can lead to gene knock-out in mitotic cells, HDR is utilized to introduce mutations into the genome (Hsu et al., 2014). We aimed to mutate wild-type APP to a Swedish APP and APP E693Δ mutation in a HCS iPSC line by delivery of an episomal plasmid and a single-stranded oligonucleotide (ssODN) as repair template (Ran et al., 2013). Due to low editing efficiencies in iPSCs, we were not able to generate clones in the course of this study. However, isogenic lines will prove invaluable to research mutation specific effects and thus should be included in further studies.

4.3 Conclusions

In the first part of this thesis we aimed to gain a deeper understanding of metabotropic NMDAR signaling in LTD and Aβ-mediated synaptic loss. We showed that blocking the NMDAR glutamate binding site but not Ca^{2+}-influx prevents loss of dendritic spines. Further, p38 MAPK activation

mediated spine shrinkage and synaptic loss is independent of G-protein signaling. We argue that prevention of Aβ binding to NMDAR could be a potential therapeutic target to prevent memory loss. However, long term treatment with NMDAR antagonists will most likely have strong side effects. CP-101606 a selective GluN2B glutamate antagonist has been shown to lead to dose-dependent amnesia (Nutt et al., 2008). Thus, manipulation of the metabotropic signaling function of the NMDAR receptor possibly is a better strategy, such as targeting of the NMDAR-CTD. Another approach is reduction of Aβ. AD patients treated with antibodies against aggregated Aβ displayed slower rates of cognitive decline compared to placebo group (Sevigny et al., 2016). This effect could depend on the prevention of spine loss, yet further research in this direction needs to be conducted.

The second part of this thesis focused on the development of a human iPSC based model with the purpose to better understand pathological changes in mitochondria, in an early phase of AD. We discovered that neuronal cells derived from some, but not all, AD patients exhibit higher ROS generation and increased levels of mitochondrial oxphos complex proteins. Further, these changes were independent of Aβ levels. We argue that aberrant mitochondrial ROS production leads to increased levels of mtDNA and DNA damage and worsens mitochondria dysfunction. Furthermore, we hypothesize that Aβ accumulation over the course of ageing may further impact mitochondria function and failure of compensatory mechanisms is followed by reduced production of ATP. This ultimately causes synaptic and cellular dysfunction and neuronal death (Grimm et al., 2015).

Functional interplay between NMDARs and mitochondria has mostly been shown in regard to calcium signaling and influx. Neurons treated with Aβ and NMDA were found to have higher intracellular and mitochondrial Ca^{2+} levels, which led to depolarization of mitochondrial membranes (Ferreira et al., 2015). Increased and prolonged Ca^{2+} levels were found to lead to excitotoxicity, in part due to increased ROS production and could be abolished by NMDAR inhibition (Stanika et al., 2010). Further, altered Ca^{2+} homeostasis led to cell death via a mitochondrial-dependent mechanism (Schinder et al., 1996). Modest Caspase-3 activation, a protein involved in apoptosis, by mitochondrial release of cytochrome-c was found to be necessary for LTD and AMPAR internalization, yet this mechanism could be blocked by APV, EGTA and treatment with inhibitors of intracellular Ca^{2+} (Li et al., 2010b). Further caspase-3 activity was found to correlate with onset of memory decline in an AD mouse model (D'Amelio et al., 2011). We observed synaptic loss mediated by Ca^{2+}-independent LTD-like mechanism, which activated p38-MAPK. P38 was found to activate Caspase3 cleavage in apoptosis (Zhuang et al., 2000). However, it remains to be discovered if both LTD mechanisms require Caspase-3 activation for AMPAR internalization and to which extend mitochondria ROS and aberrant mitochondrial function influence this process in human AD pathology.

The presented work will be instrumental to elucidate methods of synaptic loss and memory decline in AD and decipher the basic mechanism of LTD and synaptic loss. We highlighted the importance of the recently discovered metabotropic NMDAR signaling function in AD, which could lead to new therapeutic approaches. In the second line of research, we show dysregulation of mitochondria and

increased ROS levels in AD patient iPSC-derived neurons and emphasize the importance of mitochondrial dysfunction in an early, presymptomatic phase of AD.

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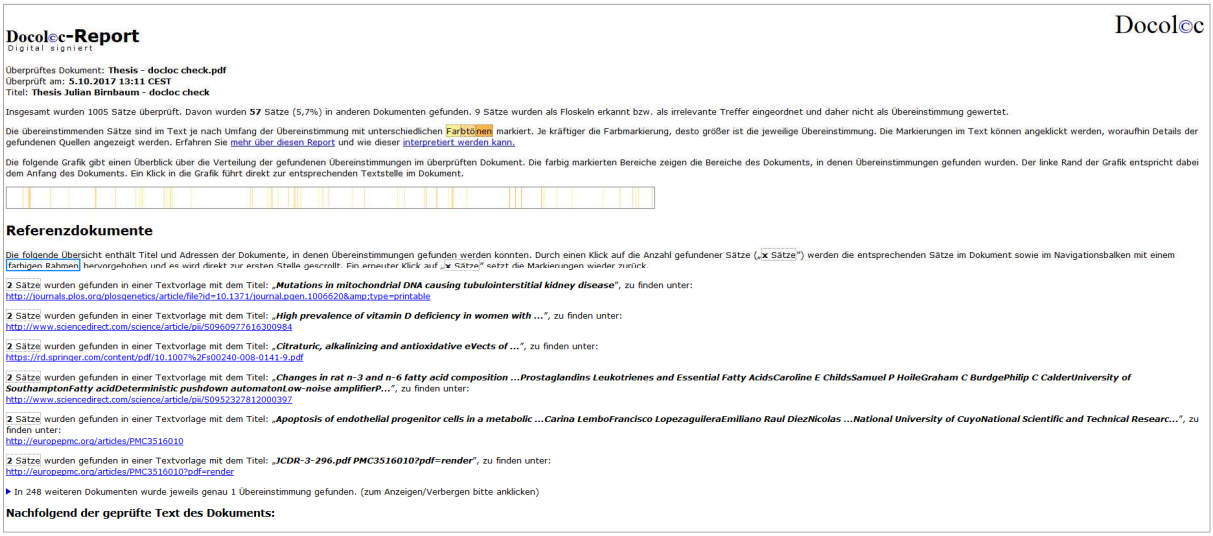

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